CERTAIN ASPECTS OF AMINO ACID NUTRITION IN LACTIC ACID BACTERIA

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CERTAIN ASPECTS OF AMINO ACID NUTRITION IN

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PREFACE

The microbiological assay technique has developed rapidly during the last few decades and has become an indispensable research tool in vitamin and amino acid chemistry. Simultaneously with the development and ever widening applicability of the technique, the number of problems and complications connected with the principles involved has increased progressively. This has caused more intense studies over a wider and wider range, covering the practical as well as the theoretical aspects of the various problems. These studies have greatly increased our knowledge of cellular metabolism and several new factors with important applications to higher forms of life as well as to microorganisms have been revealed.

One of the most recent problems encountered in microbial nutrition is the metabolic interrelationships between different amino acids in the medium. A steadily increasing number of these interrelationships have been reported during the last few years, indicating a very complex relationship, indeed. Not only are these amino acid interrelationships of a definite practical significance in the application of microbiological assay methods, but they undoubtedly also represent a little studied, but fundamentally very important, phase of amino acid metabolism.

This present study was planned to investigate one specific type of these interrelationships the goal being to improve general assay conditions as well as to obtain information about this phase of the amino acid nutrition of lactic acid bacteria.

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INTRODUCTION

The family Lactobacteriaceae is composed of gram-positive rods and chain-forming cocci. They generally grow best under anaerobic conditions, and ferment sugars with production of considerable quantities of lactic acid. They are widely distributed in nature.

The Lactobacteriaceae is divided into two tribes and seven genera as follows:

TRIBE	GENUS	MAJOR CHARACTERISTICS
Streptococceae	.....	Chain-forming, highly fermentative cocci.
	Diplococcus	Cells usually in pairs, aerobic species soluble in bile.
	Streptococcus	Cells usually in chains, insoluble in bile.
	Leuconostoc	Spherical to pointed cells differing from above genera in that both acid and gas is produced during fermentation.
Lactobacilleae	.....	Nonmotile rods, often long and slender.
	Lactobacillus	Lactic acid produced, catalase absent.
	Microbacterium	Catalase positive, lactic acid formers.
	Propionibacterium ...	Fermentation products are propionic and acetic acids, and CO_2.
	Butyribacterium	Fermentation products are butyric and acetic acid, and CO_2.

The lactic acid bacteria have been a subject of great attention since the beginning of bacteriology, mainly because of their importance in the food and fermentation industries. Some of them are essential for the production of certain foods and beverages, whereas others are

detrimental. In recent years the industrial applications of the lactic acid bacteria have been extended farther, with the discovery that the organisms can convert sucrose into high yields of polysaccharides, which upon partial hydrolysis yield effective plasma substitutes. It has also been found that some of the lactic acid bacteria produce potent antibiotic substances (1, 2, 3). These may never compete with penicillin or the other well-known antibiotics in human therapy, but may be of potential importance in the control of certain types of industrial or food spoilages.

The greatly increased attention the lactic acid bacteria have acquired during the last 10 - 15 years is, however, not due only to their new industrial applications, but also to their increasing importance as research tools in the study of nutrition and metabolism. These studies have proved very productive both in enlarging our fundamental knowledge in the fields of biochemistry and nutrition, and in improving the nutrition of higher forms of life, particularly of man.

Based on our increasing knowledge of the nutrition of the lactic acid bacteria, the microbiological assay technique for vitamins and amino acids has developed rapidly during the last decade. The practical application of such assays was first reported by Snell and Strong in 1939 (4), and they are now among the most important analytical methods in protein and vitamin chemistry (5, 6, 7).

The biochemistry of the lactic acid bacteria is very complex from the standpoint of their nutritional requirements as well as from the standpoint of interrelationships between the required nutrients. Several workers have reviewed different aspects of the nutrition of lactic acid bacteria (8, 9, 10, 11, 12). The following brief summary is based on recent reviews by Snell (13) and Hendlin (14).

Energy Sources:

Soluble carbohydrates, and especially glucose, are the principal source of energy for these organisms. Some of them can also use non-carbohydrate materials, e.g., citric acid, as energy source. The fermentation always yields lactic acid, but different organisms produce different amounts. Homofermentative organisms convert as much as 95% of the utilized hexoses to lactic acid (e.g., Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus plantarum, Streptococcus faecalis), whereas heterofermentative organisms, in addition to lactic acid, form acetic acid, ethanol, CO_2 and other products in relatively large amounts (Lactobacillus gayonii, Lactobacillus lycopersici, Leuconostoc mesenteroides).

Both homofermentative and heterofermentative organisms ferment pentoses to one mole of lactic acid and one mole of acetic acid. The mechanism of this fermentation is not yet completely understood, but is being investigated (15, 16).

Carbon and Nitrogen Requirements:

Ammonium salts and CO_2 are stimulatory, i.e. give more growth, and may even be essential, i.e. required for growth, for the lactic acid bacteria under certain conditions, evidently by serving as sources of carbon and nitrogen for the synthesis of certain components of the medium, when these are not supplied preformed (17, 18). The stimulatory effect which has been observed even when the medium apparently contains all the known required substances, is not yet clearly understood (13).

The major portion of the required nitrogen and carbon must, however, be supplied in the form of preformed organic compounds, such as amino acids, vitamins and other growth factors, e.g., purines and pyrimidins.

Vitamins and Other Growth Factors:

Complete lists of the vitamins required by the lactic acid bacteria are given in the literature in review form with cross references to the original works (13, 5). The fact that only small quantities of the different vitamins are needed indicates clearly the catalytic role of these substances in the metabolism of the organisms. The requirements can, however, be increased, decreased or even completely eliminated by changing the amount of other components in the medium (19, 20). Several illustrations for this fact will be considered in the following:

The biotin requirement of Lactobacillus arabinosus is ten times higher in aspartic acid free media than in media where ample amounts of this amino acid are supplied. The suggestion that biotin is required for the synthesis of aspartate has been confirmed by tracer studies (19). The biotin and aspartic acid requirement is also modified by other factors such as CO_2 and oleic acid (21).

Streptococcus faecalis has been shown to grow in the absence of serine when high levels of folic acid are supplied to the medium (19, 20). Similarly, the serine requirement can be eliminated if the pyrimidine, thymine is substituted for folic acid (22).

The various forms of vitamin B_6 are especially important in amino acid metabolism. Several amino acids can be omitted from the medium for several species when ample amounts of pyridoxine, pyridoxal or pyridoxamine or their phosphorylated forms are supplied, whereas some organisms require all the common amino acids, including D-alanine when B_6 is lacking (23, 24, 25, 26). Vitamin B_6 has been shown to be involved almost exclusively in transamination reactions (19, 27).

As previously indicated, purines and pyrimidines modify the requirement for certain vitamins. The relationship is especially clear for para-amino benzoic acid, folic acid and vitamin B_{12} (19, 20). The requirement for vitamin B_{12} is also known to be related to certain nucleotides, e.g., thymidine (28), and various desoxyribosides support growth of Lactobacillus lactis and Lactobacillus leichmannii in B_{12} deficient media (29, 30). Thymine also increases the response of Leuconostoc citrovorum to folinic acid (31), and folic acid as well as formylfolic acid have a similar effect (14).

The complexity of the relationships between the different components of the medium is obvious. Undoubtedly variations in the vitamin levels in the different assay media are responsible for much of the conflicting data in the literature as to the amino acid requirements of the lactic acid bacteria.

Amino Acids and Peptides:

Even with all the vitamins present in excess in the medium, the lactic acid bacteria have a very specific amino acid requirement. Generally the L-isomer of the amino acid is the required form, but specific cases of D-amino acid requirements have been reported (26), and it has also been claimed that certain D-amino acids occur naturally (32).

The number of amino acids required varies from species to species. With all vitamins supplied, Lactobacillus arabinosus, for example, requires only eight to ten amino acids, while Leuconostoc mesenteroides needs at least 17 of the 18 common amino acids for growth. There are also great variations as to the amount of amino acids necessary for growth. These variations are not only dependent on the vitamin levels

in the medium, but also on the levels of the other amino acids.

In 1948, Brickson et al. (33) described several cases of competitive antagonism between amino acids. Limiting amounts of glutamic acid, for example, were not utilized properly by Lactobacillus arabinosus in the presence of high levels of aspartic acid. If the aspartate level was lowered, or if asparagine was substituted for aspartate, utilization of glutamate took place normally in this organism. In similar manner the utilization of limiting valine and leucine was inhibited by isoleucine in both Lactobacillus arabinosus and Leuconostoc mesenteroides. The latter relationship has also been observed in Lactobacillus lactis (14). A mutual antagonism between serine and threonine has also been reported for several species (34).

In contrast to the above antagonism is the amino acid interdependence reported by Sirny et al. in 1951 (35). They showed that in order to utilize limiting amounts of arginine, Leuconostoc mesenteroides required high levels of proline in the medium, and in order to utilize limiting proline, it required high levels of arginine.

Utilization of peptides by microorganisms has also received great attention the last few years. The first data all indicated that peptides are utilized less readily than the free amino acids. Later, however, several cases of growth stimulation have been reported for the lactic acid bacteria, when the amino acid is substituted by peptides, either in the form of incomplete protein hydrolysates or as the synthetic compounds (36, 37, 38, 39, 40, 41, 42, 43). Partial hydrolysates was found to overcome the interdependence, mentioned above (35), and it seems like any stimulatory effect of peptides is observed only if the organisms are grown under suboptimal conditions. Although some explanation for

the stimulatory effect have been offered, the picture is yet far from clear (36).

Inorganic ion Requirements:

The requirement for inorganic ions by the lactic acid bacteria is probably also a very complex one, but this problem has had less attention than the previous ones. The main reason for these only limited studies is undoubtedly connected with the difficulties involved in producing media sufficiently free from traces of inorganic contaminants to allow study of the ion requirement. It has been established, however, that relatively high amounts of potassium, manganese and phosphorus are required for all species. Some species also require magnesium (11) and calcium (44). The requirement for the different inorganic ions does not seem to be very specific, since it has been shown for example, that rubidium replaces potassium for Streptococcus faecalis and that magnesium, strontium and calcium at least partially overcome the manganese requirement of Lactobacillus arabinosus (45). In the utilization of pantothenic acid by Lactobacillus arabinosus a possible requirement for sodium has also been observed (46). Antagonisms between inorganic ions have also been reported (47).

Other Factors Influencing the Nutrition of Lactic Acid Bacteria:

Incubation temperature. Both the rate of growth and the amount of growth is greatly affected by temperature, and rather marked changes in the nutritional requirements have also been noted when the incubation temperature is varied as little as one to two degrees (48, 49). The optimal temperature range for the whole family of organisms is very wide, some species being able to grow at temperatures slightly above the freezing point, while others have optimal growth temperatures approaching

those of true thermophiles.

Hydrogen ion concentration. The optimal pH range for the family is also very wide, varying from 4.0 for some species to 9.5 for others. The fact that the bacteria are able to produce enough acid to drastically change the pH of the system has necessitated use of strongly buffered media. The direct effect of pH on the nutrition has not been studied to any great extent, but it is recognized as an important factor. The above-mentioned amino acid antagonisms and interdependences, for example, are all partially relieved by lowering the pH from 7 to 6 (20, 35). There is also evidence that a relationship exists between pH, temperature and the CO_2 requirement, since both pH and temperature exert a direct effect on the CO_2 tension.

Oxidation-reduction potential. Lowry and Bessey (50) reported that high oxygen tension had an inhibitory effect on the assay organism in their microassay for riboflavin, in which they used an assay tube volume of only 0.2 ml. In conventional semi-micro assays, however, the larger volume combined with conditions resulting from the breakdown of carbohydrates during autoclaving brings about favorable oxidation-reduction potentials. Thus it has not been considered necessary to control the O/R potential of the medium. Recently, however, several reports have occurred in the literature which implicate this factor as one of the causes for assay variations, since it is known to alter the nutrition of the organisms considerably (51, 52, 53, 54). A more intense study along these lines is undoubtedly in progress.

Sterilization conditions. The most frequently used method for sterilization of the assay medium is autoclaving at 120° C. It is

known that the autoclaving time has some effect on the assay results. Some amino acids are destroyed during autoclaving, e.g., cystine, lysine and histidine (55), presumably by reacting with equimolecular amounts of aldoses in the medium (56, 57, 58) to give a product which is described as a Schiff base (59), and which gives the medium a dark brown color. This reaction has been known for a long time as the Maillard reaction (60). Conversely, autoclaving may also have a stimulatory effect on the growth of the lactic acid bacteria (61, 62). It has been suggested that this stimulation is due to formation of caramelization products, which act as growth factors for the organisms. It has been shown in support of this suggestion, that caramelized dextrose, pyruvic acid and acetaldehyde are stimulatory to growth of the lactic acid bacteria (63). Thus, both these factors have to be considered when the autoclaving time is determined.

It is evident that all the variables complicate the application of the microbiological assay techniques, and that they must be given cognizance when the assay results are evaluated. It also seems evident that there is too much lacking in our present knowledge to allow an intelligent correlation of the different factors and an analysis of their specific effects. Further studies will, however, undoubtedly widen the knowledge and make possible a more universal application of the hitherto unused and unavailable potentialities of the lactic acid bacteria as research tools.

GENERAL PROCEDURES

Assay Organisms:

The studies to be described in this thesis were limited to the following six lactic acid bacterial species, which were considered representative of those most commonly used in microbiological assay work:

Leuconostoc mesenteroides P-60	(ATCC 8042)
Leuconostoc citrovorum	(ATCC 8081)
Lactobacillus arabinosus 17-5	(ATCC 8014)
Lactobacillus delbrückii 3	(L. d. III Henneberg)[1]
Lactobacillus brevis	(ATCC 8257)
Streptococcus faecalis R	(ATCC 8043)

The organisms were carried on agar stab cultures under refrigeration and transferred approximately every month. The agar medium (Appendix A) was made up approximately every six months.

For use, the organisms were transferred into liquid medium (Appendix A) and incubated at 37° C. After 12 to 24 hours incubation, the actively growing bacteria were centrifuged down, and the supernatant discarded. The cells were resuspended in 0.9% KCl solution and added dropwise to the assay tubes by means of a syringe.

Basal Medium:

The medium used in this work (Appendix B) was a modification of that recommended by Henderson and Snell (64). Instead of using salts of both sodium and potassium, potassium salts were substituted for all sodium salts, providing a medium that is free of added sodium. Such an

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[1] Described by Sirny *et al.* in J. Nutrition, 41:383, (1950).

"all-K" modification has been used by Sirny (46), who reported it to be superior to the normal Henderson-Snell medium under many conditions.

This uniform medium was used as such in all studies in which Leuc. mesenteroides, L. arabinosus and S. faecalis were used as assay organisms. For Leuc. citrovorum the medium was supplemented with liver concentrate[2] at a level recommended by Steele et al. (65), and for L. delbrückii-3 it was supplemented with 200 ug. $CaCl_2$ per tube.

For L. brevis an entirely different medium was used, since this organism prefers arabinose to glucose as its energy source. This medium (Appendix B) is an "all-K" modification of the medium proposed by Dunn et al. (67)

General Techniques:

Preparation of assays. The general methods used in this work were those described by Henderson and Snell (64), with certain modifications only in more complex experiments. These modifications will be considered in connection with the specific experiments in which they were used.

Assay racks containing 60 tubes in 10 rows were employed, and the different experiments were set up as follows:

Standard solution of the limiting amino acids was added in graded amounts in each row, so that the six tubes received 0.0 -0.2 - 0.4 - 0.6 - 0.8 - 1.0 ml. respectively. Water was then added so that the total volume in each tube was 1 ml. 1 ml. of the basal medium, containing an excess of all components except the limiting amino acid was finally added to give a total volume of 2 ml. All additions were made with a Cannon Automatic Dispenser (68).

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[2]Reticulogen, Eli Lilly and Co.

Sterilization. During the first part of the work the effective autoclaving time was subject to variations, and it was felt that the effective sterilization conditions were occasionally too long. A relatively short, well-controled sterilization was desired, and the following procedure was adopted to standardize conditions as much as possible.

Previous to use, the autoclave was heated for several minutes at approximately 15 lbs. pressure. The pressure was then reduced rapidly and the assay racks put in. By this means, the temperature could be brought up to the desired 121° C. in a minimum of time. Exactly 5 minutes after this temperature was attained, the steam was shut off and the pressure brought down within one minute. The racks were immediately removed and allowed to cool outside the autoclave.

Incubation. After inoculation the racks were incubated at a constant temperature of 37 ± 1° C. Incubation time varied some, since normal procedure was to titrate the third day after inoculation, neglecting the time of the day the inoculation and titration were performed. Thus the average time range for incubation was 64 - 80 hours.

In large assays there might even be as much as a 6 to 8 hours time lapse between the titration of the first and the last rack of the same assay. Generally, however, all racks were removed from the incubator when titration started, and since, furthermore, each rack contained a standard row for checking such variations, the possible effect of the relatively small differences in incubation time could be neglected within each assay.

Titration. The amount of growth in each tube was determined by titration of the produced acid with approximately 0.05 N KOH. An electrometric method was used, applying a calomel half cell as reference

electrode and a quinhydrone electrode as indicator. The zero point of the galvanometer, which indicated the end point of the titration, was set at pH = 7.3, previous to each titration. A Cannon Automatic Titrator was used (68), and the amount of base required was recorded as titration counts, 100 counts corresponding to approximately 4 ml. of base.

As already discussed in the introduction, and as shown above, there are several variables in this kind of work, and these variables and the degree to which they are controlled must be considered in evaluating the results. It was felt that by following a previously planned general procedure and deviating from it as little as possible in each different experiment, some of the variables would be reduced. Thus it was also felt that the results could be considered with the same confidence as is given to any analytical method.

PART I

A SURVEY OF AMINO ACID INTERDEPENDENCES

Purpose of Experiment:

This work was undertaken to study further the amino acid interdependence reported by Sirny *et al.* in 1951 (35). As already mentioned, they showed that *Leuc. mesenteroides* in order to utilize limiting amounts of arginine, requires high levels of proline in the medium, and in order to utilize limiting amounts of proline, requires high levels of arginine. They demonstrated evidence that an interconversion between the two amino acids was unlikely, and the interdependence was, therefore, suggested to be due to a difficult peptide synthesis:

> "arginine and proline are involved together in the formation of a compound or compounds, the synthesis of which is difficult for the organism."

In the original paper the question was raised whether or not the arginine-proline relationship was a separate, specific one, or if similar relationships existed for other amino acid combinations and with other organisms. The present studies were undertaken to investigate that question.

Experimental:

The research plan was designed as a complete survey of the specific effects of lowered amounts of each of the seventeen amino acids on the utilization of limiting amounts of the individual amino acids, described as required by the six organisms listed above.

To illustrate the organization of an individual experiment the

"Alanine-Amino Acid Interdependence" experiment is shown below as a typical example:

Rack 1

Rows:	1	2	3	4	5	6	7	8	9	10
	All 17 amino acids high.	Low arginine	Low aspartic acid	Low cystine	Low glutamic acid	Low glycine	Low histidine	Low isoleucine	Low leucine	

Rack 2

Rows:	1	2	3	4	5	6	7	8	9	10
	All 17 amino acids high.	Low lysine	Low methionine	Low ph-alanine	Low proline	Low serine	Low threonine	Low tyrosine	Low tryptophan	Low valine

The eighteenth amino acid, alanine, was added as standard at the level of 20 ug. per ml. Thus, the six tubes in each row contain, in order, 0, 4, 8, 12, 16, and 20 ug. of DL-alanine. All tube volumes were adjusted to one ml. with water. The previously prepared amino acid mixes (each containing 16 amino acids at high level, one at low level and with alanine missing) were added to the "all-K" modification of the Henderson-Snell sugar-buffer mix, and the pH of each individual solution adjusted to 7.0. This final basal medium was added at the level of one ml. per tube.

As to the concentrations of the amino acids in the medium, the Henderson-Snell medium was used as a basis. The term "high" refers to

the prescribed concentration in this medium, while the term "low" refers to a concentration of 1/10 of the prescribed or "high" amount. It should be noted that the "low" amount still represents an excess of the amino acid, from two to ten times as high as the amount used when the amino acid is limited for establishing a standard curve.

The "high" and "low" concentrations, the "standard" concentrations, and the different organisms used in the assaying of each amino acid are given in table I.

It was felt that the most serious source of variation in this experimental procedure was the adjustment of pH, between the eighteen different solutions within each assay as well as between assays. This source of variation was partially evaluated, however, by the use of several organisms in each experiment. If one effect, e.g., high blanks, increased or decreased overall growth, was observed with all the organisms used, this was considered a pH effect unless a repeated experiment proved otherwise. If an effect showed up for only one or two organisms, while the other organisms on the same medium gave growth curves identical to the standard curves, this was considered a true effect of the "low" amount of the one amino acid on the utilization or synthesis of the limiting amino acid.

Results:

The results obtained are conveniently classified in two main groups with two subgroups in each, according to the four fundamentally different growth responses to the test conditions.

The four different groups are:

1. Effects on the utilization of the limiting amino acid.

a. Inhibition of utilization by high amounts of another amino acid.

b. Inhibition of utilization by low amounts of another amino acid.

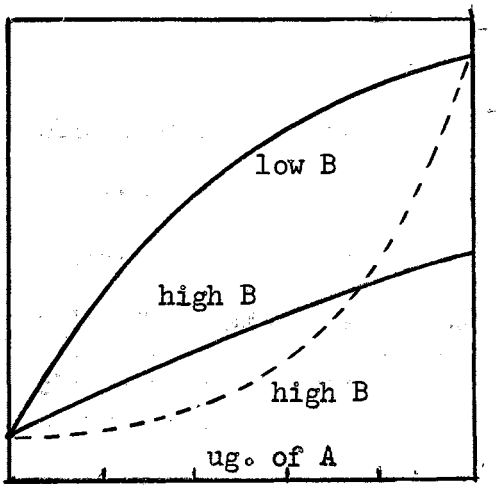
2. Effects on the synthesis of the limiting amino acid.

a. Inhibition of synthesis by high amounts of another amino acid.

b. Inhibition of synthesis by low amounts of another amino acid.

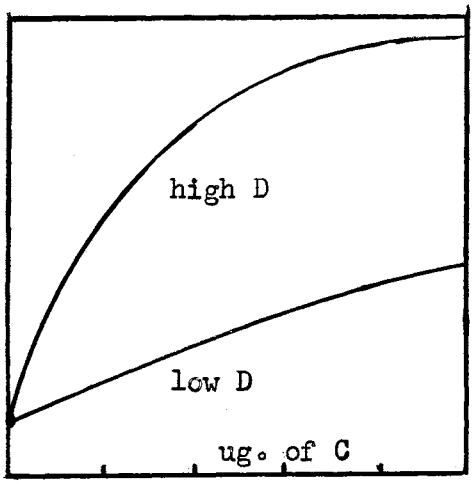
An explanation of the above groups follows, without any attempt to explain the mechanism involved in each specific case at this time:

1 a. <u>The inhibition of utilization by high amount of another amino acid</u> is a rather well-known interrelationship among amino acids, and is commonly termed a competitive inhibition. Lowering the antagonistic amino acid in the medium results in a more efficient utilization of the limiting amino acid, A. In other words, the amino acid B prevents the optimal utilization of A. The effect may show up only at the lower levels of A (dotted line on graph) or it may be a consistant inhibition at all levels of A within the limits of the concentration of the standard curve (solid line on the graph).

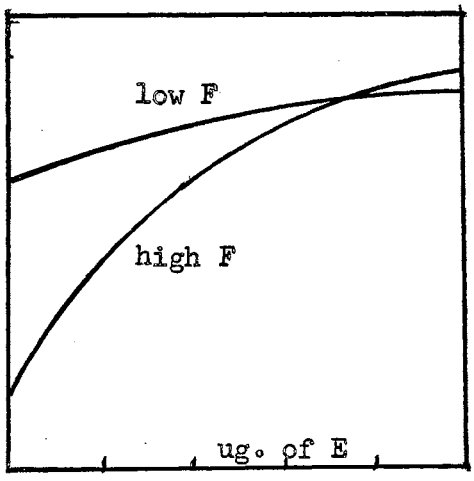


Several examples of this type of inhibition have been reported in the literature. In this survey 76 different interrelationships of this kind were revealed.

1 b. Inhibition of utilization by low amount of another amino acid in the medium. This group consists of the interrelationships of the type reported by Sirny et al. and which was termed an amino acid interdependence. The interdependence is manifested as a decreased efficiency in the utilization of an amino acid C, when the concentration of another amino acid D in the medium is lowered. In the survey 161 interdependences of this kind were found. Of these 13 may be termed reversible to indicate a reciprocal interrelationship, in which high levels of D are required for optimal utilization of limiting amounts of C, and high levels of C are required for optimal utilization of limiting amounts of D. The other interrelationships in this group were not found to be reversible, i.e., high levels of D were required for optimal utilization of C, but the converse was not true.

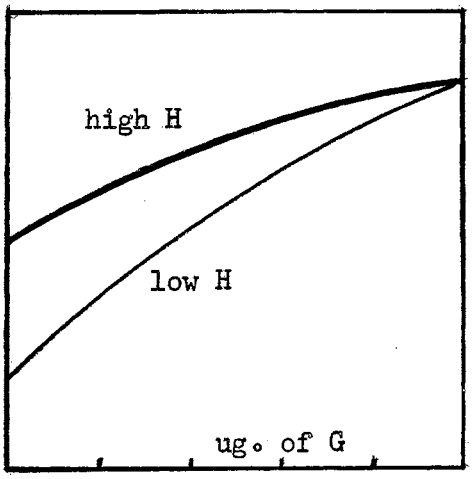


2 a. Inhibition of synthesis by high amount of another amino acid in the medium. This effect is revealed as a high blank on the growth curve when the level of the inhibiting amino acid F is lowered. In other words, in the presence of only small amounts of F the organism is able to synthesize the limiting amino acid E. The synthesis is inhibited when higher levels of F are supplied, which is indicated by the lower



blanks in the standard curve under these conditions. A total of 43 cases of this kind of interrelationship were found.

2 b. Inhibition of synthesis by low amount of another amino acid in the medium. This picture is the reverse of that above. When the level of the amino acid H is decreased, the blanks are lowered as compared to those in the standard curves where H is present at a high level. Thus, for the synthesis of the limiting amino acid G, a high level of H is required. The effects of this type of interrelationships were not as pronounced as those in the other groups, but they are considered sufficiently striking to be included in this summary. Only 18 cases were found.



The various interrelationships revealed in this survey are summarized by name only in Tables II, III, IV and V. Because of the extensive nature of the survey and the large number of interrelationships found, it is not considered feasible to present the supporting data for each individual case. It should be pointed out, however, that the individual cases are, for the most part based on observations made in single experiments. It should further be noted that the interrelationships vary considerably in degree, from very slight to very profound effects of the modifying amino acid on the utilization of the limiting one.

Thus, it may be that certain of the interrelationships reported in the tables may, on further experimentation, prove to be artifacts or

relatively insignificant effects. Conversely, it may be found that other interrelationships have escaped attention, either because of the experimental design or because of the limited nature of these preliminary studies.

In order that the results presented may be viewed in the proper perspective as to the number of the different interrelationships revealed, it seems desirable to re-emphasize that with each organism, only those amino acids required by that particular organism could be studied. The number of amino acids was as follows for the different organisms:

Leuconostoc mesenteroides: all 18 amino acids.

Leuconostoc citrovorum: 14 amino acids.

Lactobacillus delbrückii-3: 9 amino acids.

Lactobacillus arabinosus: 6 amino acids.

Streptococcus faecalis: 11 amino acids.

Lactobacillus brevis: 3 amino acids.

Discussion:

Several reviewers have pointed out the necessity of controlling the concentrations of the various vitamins in the basal medium and consider these values when the assay results are evaluated. The surprisingly large number of amino acid interrelationships found in this study clearly indicates that similar care must be taken in formulating the amino acid concentrations in the basal media, and in comparison of results from assays where different amino acid concentrations were used.

The survey has also demonstrated a distinct drawback in using essentially only one basal medium for assays for all 18 amino acids with several organisms. Certain modifications have already been recommended

in specific cases, e.g., asparagine is substituted for aspartic acid in the medium used in assays for glutamic acid, a lowered amount of threonine is used in assays for serine. A much more extended use of such modifications seems unavoidable in view of the large quantity and complexity of the amino acid interrelationships reported above.

Further studies indicated as a consequence of these preliminary observations are, firstly, verification of the individual cases reported here, and secondly, investigation of other factors modifying each specific case. Some such further studies have already been conducted, and these are described in subsequent sections of this thesis.

TABLE I

Summary of Organisms and Conditions Used in the Interdependence Studies

Amino acid	Conc. of standard ug./ml. of the L-form	"high" ug./tube	"low" ug./tube	Assay organisms
DL-alanine	20	2000	200	L. mes., L. citr.
DL-aspartic acid	50	2000	200	L. mes., L. brevis
L-arginine.HCl	20	400	40	L. mes., L. citr. L. d.-3, S. faec.
L-cystine	5	200	20	L. mes., L. citr.
DL-glutamic acid	60 (50*)	2000	200	L. mes., L. citr., L. d.-3, L. arab., S. faec.
Glycine	10	200	20	L. mes., L. citr., L. brevis
L-histidine.HCl	5	200	20	L. mes., L. citr., L. d.-3, S. faec.
DL-isoleucine	15	400	40	L. mes., L. citr., L. d.-3, L. arab., S. faec.
L-leucine	15	200	20	L. mes., L. citr., L. d.-3, L. arab., S. faec.
L-lysine. HCl	30	400	40	L. mes., S. faec.
DL-Ph-alanine	10	400	40	L. mes., L. citr., L. d.-3, L. arab.
DL-methionine	8	400	40	L. mes., L. citr., S. faec.
L-proline	10	200	20	L. mes., L. citr.
DL-serine	20	400	40	L. mes., L. d.-3, L. brevis
DL-threonine	15	400	40	L. mes., L. citr. S. faec.
DL-tryptophan	6	400	40	L. mes., L. arab., S. faec.
L-tyrosine	10	200	20	L. mes., L. citr., L. d.-3, S. faec.
DL-valine	20	400	40	L. mes., L. citr., L. d.-3, L. arab., S. faec.
DL-asparagine*	--	500	50	

*One assay of glutamic acid was run with asparagine instead of aspartic acid (33) and with the glutamic acid standard at 50 ug. per ml.

TABLE II

Inhibition of the Utilization of the Limiting Amino Acid
by High Amounts of A Modifying Amino Acid

Organism	Limiting Amino Acid	Modifying Amino Acid	Limiting Amino Acid	Modifying Amino Acid
Leuc. mes.	Arginine	Cystine	Cystine	Valine
	Leucine	Valine	Threonine	Serine
	Glutamic Acid=	Aspartic Acid	Glycine	Alanine
	Glutamic Acid=	Glycine	Glycine	Cystine
	Glutamic Acid=	Proline	Glycine	Serine
	Glutamic Acid=	Serine	Glycine	Valine
	Glutamic Acid=	Threonine	Serine	Methionine
	Glutamic Acid*	Methionine	Serine	Tryptophane
	Glutamic Acid*	Ph-alanine		
Leuc. citr.	Isoleucine	Cystine	Cystine	Proline
	Isoleucine	Tyrosine	Cystine	Threonine
	Leucine	Tryptophan	Cystine	Tyrosine
	Glutamic Acid=	Aspartic Acid	Threonine	Serine
	Glutamic Acid=	Glycine	Threonine	Valine
	Glutamic Acid=	Histidine	Glycine	Cystine
	Glutamic Acid=	Isoleucine	Glycine	Isoleucine
	Glutamic Acid=	Leucine	Glycine	Leucine
	Glutamic Acid=	Lysine	Glycine	Lysine
	Glutamic Acid=	Methionine	Glycine	Tyrosine
	Glutamic Acid*	Alanine	Glycine	Tryptophan
	Cystine	Methionine	Glycine	Valine
	Cystine	Ph-alanine		
L. d.-3	Ph-alanine	Aspartic Acid	Glutamic Acid=	Aspartic Acid
	Ph-alanine	Arginine	Glutamic Acid=	Arginine
	Ph-alanine	Cystine	Tyrosine	Arginine
	Histidine	Glycine	Tyrosine	Ph-alanine
	Leucine	Cystine	Tyrosine	Tryptophan
	Valine	Glycine	Serine	Alanine
	Valine	Proline		
L. arab.	Ph-alanine	Histidine	Glutamic Acid=	Glycine
	Isoleucine	Leucine	Glutamic Acid=	Leucine
	Isoleucine	Valine	Glutamic Acid=	Serine
	Glutamic Acid=	Aspartic Acid	Glutamic Acid=	Tyrosine
	Glutamic Acid=	Arginine		
L. brevis	Serine	Alanine	Serine	Aspartic Acid

Organism	Limiting Amino Acid	Modifying Amino Acid	Limiting Amino Acid	Modifying Amino Acid
S. faec.	Leucine	Ph-alanine	Glutamic Acid*	Methionine
	Leucine	Tyrosine	Tyrosine	Lysine
	Valine	Serine	Threonine	Serine
	Glutamic Acid=	Aspartic Acid	Tryptophan	Aspartic Acid
	Glutamic Acid*	Alanine		
	Glutamic Acid*	Histidine		

*Assay of glutamic acid with asparagine substituted for aspartic acid.
=Assay of glutamic acid with aspartic acid in the medium.

TABLE III

Inhibition of the Utilization of the Limiting Amino Acid
by Low Amounts of A Modifying Amino Acid

Organism	Limiting Amino Acid	Modifying Amino Acid	Limiting Amino Acid	Modifying Amino Acid
Leuc. mes.	≠Arginine	Glutamic Acid=	Cystine	Ph-alanine
	≠Arginine	Glycine	≠Proline	Cystine
	≠Arginine	Lysine	Proline	Glutamic Acid=
	≠Arginine	Serine	Proline	Glycine
	≠Arginine	Threonine	Proline	Histidine
	≠Arginine	Proline	Proline	Lysine
	Ph-alanine	Glycine	≠Proline	Threonine
	Ph-alanine	Proline	Methionine	Arginine
	Ph-alanine	Serine	Methionine	Proline
	≠Ph-alanine	Threonine	Methionine	Serine
	≠Ph-alanine	Tyrosine	Methionine	Threonine
	Histidine	Glutamic Acid	Lysine	Threonine
	Histidine	Threonine	≠Threonine	Tryptophan
	Leucine	Arginine	Aspartic Acid	Arginine
	Leucine	Proline	Glycine	Glutamic Acid
	Leucine	Threonine	≠Glycine	Histidine
	Valine	Methionine	Serine	Glycine
	Tyrosine	Glycine	Serine	Isoleucine
	Tyrosine	Proline	Serine	Leucine
	≠Tyrosine	Serine	Serine	Lysine
	Tyrosine	Threonine	Serine	Cystine
	Glutamic Acid*	Asparagine	Serine	Valine
	Glutamic Acid*	Cystine	Alanine	Arginine
	Glutamic Acid*	Glycine	Alanine	Lysine
	Cystine	Leucine	Alanine	Ph-alanine
	Cystine	Methionine	Alanine	Proline
Leuc. citr.	Arginine	Isoleucine	Valine	Methionine
	≠Arginine	Leucine	Tyrosine	Serine
	Arginine	Valine	Glutamic Acid*	Asparagine
	Ph-alanine	Glutamic Acid	Cystine	Alanine
	Ph-alanine	Isoleucine	Threonine	Glutamic Acid
	Ph-alanine	Valine	Threonine	Ph-alanine
	Histidine	Glutamic Acid	Glycine	Proline
	Leucine	Glutamic Acid	Alanine	Glutamic Acid
	Leucine	Proline	Alanine	Glycine
	Leucine	Threonine	Glutamic Acid=	Cystine
	Leucine	Tyrosine		

Organism	Limiting Amino Acid	Modifying Amino Acid	Limiting Amino Acid	Modifying Amino Acid
L. d.-3	Arginine	Cystine	Valine	Glutamic Acid
	Arginine	Glutamic Acid	Valine	Lysine
	Arginine	Isoleucine	≠Valine	Serine
	Arginine	Leucine	≠Valine	Tyrosine
	Arginine	Tryptophan	Tyrosine	Glutamic Acid
	Arginine	Tyrosine	Tyrosine	Alanine
	Arginine	Valine	Tyrosine	Aspartic Acid
	Ph-alanine	Glutamic Acid	Tyrosine	Cystine
	Ph-alanine	Serine	Tyrosine	Glycine
	Histidine	Cystine	Tyrosine	Lysine
	Histidine	Glutamic Acid	≠Tyrosine	Serine
	Histidine	Lysine	Tyrosine	Proline
	Histidine	Proline	Glutamic Acid*	Asparagine
	Histidine	Serine	Glutamic Acid*	Cystine
	Histidine	Tryptophan	Glutamic Acid*	Isoleucine
	Histidine	Tyrosine	Glutamic Acid*	Tyrosine
	Isoleucine	Glutamic Acid	Serine	Cystine
	≠Isoleucine	Serine	Serine	Glutamic Acid
	Isoleucine	Tryptophan	Serine	Leucine
	Leucine	Glutamic Acid	Arginine	Serine
L. arab.	Ph-alanine	Threonine	Glutamic Acid*	Cystine
	Leucine	Glutamic Acid	Glutamic Acid*	Lysine
	Valine	Cystine	Glutamic Acid*	Proline
	≠Valine	Tryptophan	Tryptophan	Isoleucine
	Valine	Tyrosine	Tryptophan	Leucine
S. faec.	Arginine	Cystine	Glutamic Acid=	Lysine
	Arginine	Leucine	Tyrosine	Cystine
	Histidine	Cystine	Glutamic Acid*	Cystine
	Isoleucine	Cystine	Glutamic Acid*	Tryptophan
	Leucine	Arginine	Methionine	Cystine
	Valine	Methionine	Threonine	Cystine
	Valine	Proline	Tryptophan	Cystine
	Glutamic Acid=	Arginine	Tryptophan	Lysine
	Glutamic Acid=	Cystine	Tryptophan	Valine
L. brevis	Aspartic Acid	Isoleucine	Serine	Glycine
	Aspartic Acid	Leucine	Serine	Histidine
	Aspartic Acid	Lysine	Serine	Isoleucine
	Aspartic Acid	Serine	Serine	Leucine
	Aspartic Acid	Valine	Serine	Lysine
	Glycine	Glutamic Acid	Serine	Ph-alanine
	Glycine	Lysine	Serine	Proline

Organism	Limiting Amino Acid	Modifying Amino Acid	Limiting Amino Acid	Modifying Amino Acid
L. brevis	Glycine	Threonine	Serine	Threonine
	Serine	Arginine	Serine	Tyrosine
	Serine	Glutamic Acid	Serine	Valine

≠ Reversible interdependences.
* Assay of glutamic acid with asparagine in the medium.
= Assay of glutamic acid with aspartic acid in the medium.

TABLE IV

Inhibition of the Synthesis of the Limiting Amino Acid
by High Amounts of A Modifying Amino Acid

Organism	Limiting Amino Acid	Modifying Amino Acid	Limiting Amino Acid	Modifying Amino Acid
Leuc. mes.	Serine	Alanine	Aspartic Acid	Tryptophan
	Serine	Cystine	Alanine	Methionine
	Serine	Tryptophan		
Leuc. citr.	Glycine	Serine	Cystine	Alanine
	Alanine	Histidine	Cystine	Arginine
	Alanine	Isoleucine	Cystine	Aspartic Acid
	Isoleucine	Tyrosine	Cystine	Glycine
	Valine	Glycine	Cystine	Ph-alanine
	Valine	Histidine	Cystine	Valine
	Valine	Leucine	Proline	Arginine
	Valine	Lysine	Proline	Ph-alanine
	Valine	Proline	Threonine	Serine
	Tyrosine	Tryptophan	Alanine	Cystine
	Tyrosine	Valine	Alanine	Glutamic Acid
	Glutamic Acid*	Arginine	Alanine	Proline
	Glutamic Acid*	Methionine	Alanine	Tryptophan
L. d.-3	Serine	Cystine	Serine	Aspartic Acid
S. faec.	Tryptophan	Aspartic Acid	Glutamic Acid*	Serine
L. brevis	Glycine	Alanine	Aspartic Acid	Methionine
	Serine	Alanine	Glycine	Aspartic Acid
	Serine	Aspartic Acid	Aspartic Acid	Alanine

* Assay of glutamic acid with asparagine substituted for aspartic acid.

TABLE V

Inhibition of the Synthesis of the Limiting Amino Acid
by Low Amounts of A Modifying Amino Acid

Organism	Limiting Amino Acid	Modifying Amino Acid	Limiting Amino Acid	Modifying Amino Acid
Leuc. mes.	Leucine	Isoleucine	Alanine	Proline
Leuc. citr.	Arginine	Glutamic Acid	Alamine	Glutamic Acid
	Leucine	Isoleucine	Alanine	Ph-alanine
	Methionine	Isoleucine	Alanine	Threonine
	Alanine	Arginine		
L. d.-3	Leucine	Isoleucine		
S. faec.	Methionine	Glutamic Acid	Lysine	Arginine
	Methionine	Isoleucine	Lysine	Cystine
L. brevis	Glycine	Glutamic Acid	Serine	Glutamic Acid
	Serine	Cystine	Serine	Glycine

PART II

STUDIES OF SPECIFIC INSTANCES OF AMINO ACID INTERRELATIONSHIPS

A. Amino Acid Inhibition of Utilization of Limiting Amounts of Glutamic Acid by Lactobacillus arabinosus.

The following experiments were conducted to investigate the possibilities for an improved assay for glutamic acid with Lactobacillus arabinosus at pH 7 under the general conditions described by Henderson and Snell (64).

A competitive inhibition between glutamic acid and aspartic acid is known, and in order to obtain acceptable assay results for glutamic acid, several modifications of the assay medium have been suggested. The most commonly employed modification is to lower the aspartic acid in the basal medium, or substitute asparagine for aspartic acid. Conducting the assay at pH 6 has also given good results, since the inhibition is markedly decreased at lower pH (69, 70, 71, 33). Heavy inoculum has been recommended for this assay (69).

In the previous survey Lactobacillus arabinosus did not respond at all to the limiting amounts of glutamic acid (standard was 60 ug./ml.) on the Henderson-Snell basal medium at pH 7. The lowering of several amino acids in the medium resulted in an increased response to glutamic acid. This however, was far from a maximal response as shown by the marked lag in the different growth curves.

The original results are shown in Fig. 1. They indicate that aspartic acid and arginine are the most active inhibitors of glutamic acid

utilization. Leucine comes next, and then serine, tyrosine, and glycine in that order, the latter three with relatively small effect.

Experimental:

In setting up these experiments a slightly different procedure was employed, which made possible several variations in the amino acid concentrations and yet permitted use of a uniform basal medium. The procedure is described below:

The following 7 solutions were prepared:

1. Arginine 1/10 normal[3] concentration, aspartic acid and leucine 1/2 normal concentration.
2. Aspartic acid 1/10 normal conc., arginine and leucine 1/2 normal conc.
3. Leucine 1/10 normal conc., aspartic acid and arginine 1/2 normal conc.
4. Arginine and aspartic acid 1/10 normal conc., leucine 1/2 normal conc.
5. Aspartic acid and leucine 1/10 normal conc., arginine 1/2 normal conc.
6. Arginine and leucine 1/10 normal conc., aspartic acid 1/2 normal conc.
7. Arginine, aspartic acid, and leucine 1/10 normal conc.

These 7 solutions were used instead of water in preparing the glutamic acid standard solutions, and also added to the racks instead of water, each solution added to the rack with the corresponding number. Thus Rack No. 1 received Solution 1 at the levels 1.0, 0.8, 0.6, 0.4, 0.2, 0.0 ml. in each row, and then glutamic acid standard (50 ug./ml.) made up in Solution 1 at the levels 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 ml. for each row. By this procedure each tube received a total volume of one

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[3]"Normal" refers to the Henderson-Snell concentration.

milliliter containing the normal level of glutamic acid standard and in addition also the varying combinations of different concentrations of arginine, aspartic acid, and leucine.

The "all-K" medium was mixed with an amino acid mixture containing 11 amino acids at the normal level and glycine, serine, and tyrosine at 1/2 the normal level. Glutamic acid, arginine, aspartic acid, and leucine were omitted. This basal medium was separated into two parts, the pH of one being adjusted to 6, and the other to 7. One ml. of the basal medium was added to each tube so that five rows in each rack were at pH 6 and five rows at pH 7.

Results:

The growth curves for the different conditions are shown in Figs. 2a, 2b, 3a, 3b, and 4. The effects of varied concentrations of the 6 modifying amino acids are rather pronounced. A satisfactory growth curve is obtained for glutamic acid at pH 7 when serine, tyrosine, and glycine are lowered to 1/2, and arginine, aspartic acid, and leucine to 1/10 of the level recommended in the Henderson-Snell medium. The effects at pH 6 are less marked, but yet clearly enough indicate that the above medium is also more optimal for glutamic acid utilization at this lower pH.

Figures 1 and 2

Response of L. arabinosus to glutamic acid under various conditions.

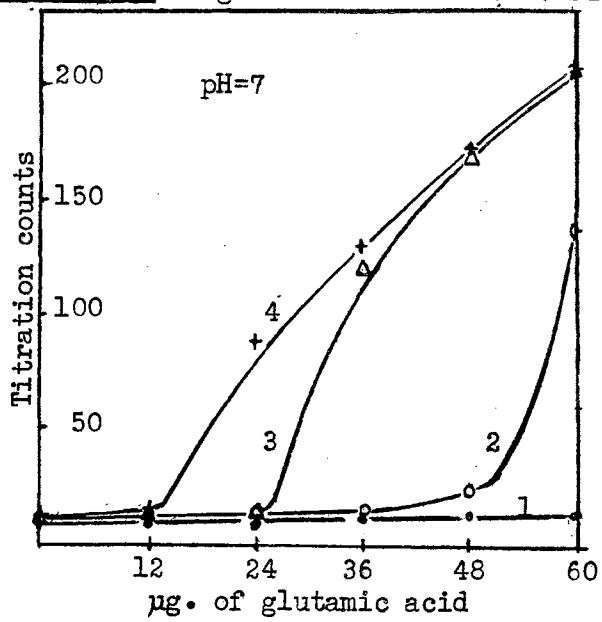


Fig.1 Original observations from the survey. (1) Normal H-S-medium, (2) low leucine, (3) low arginine, (4) low aspartic acid.

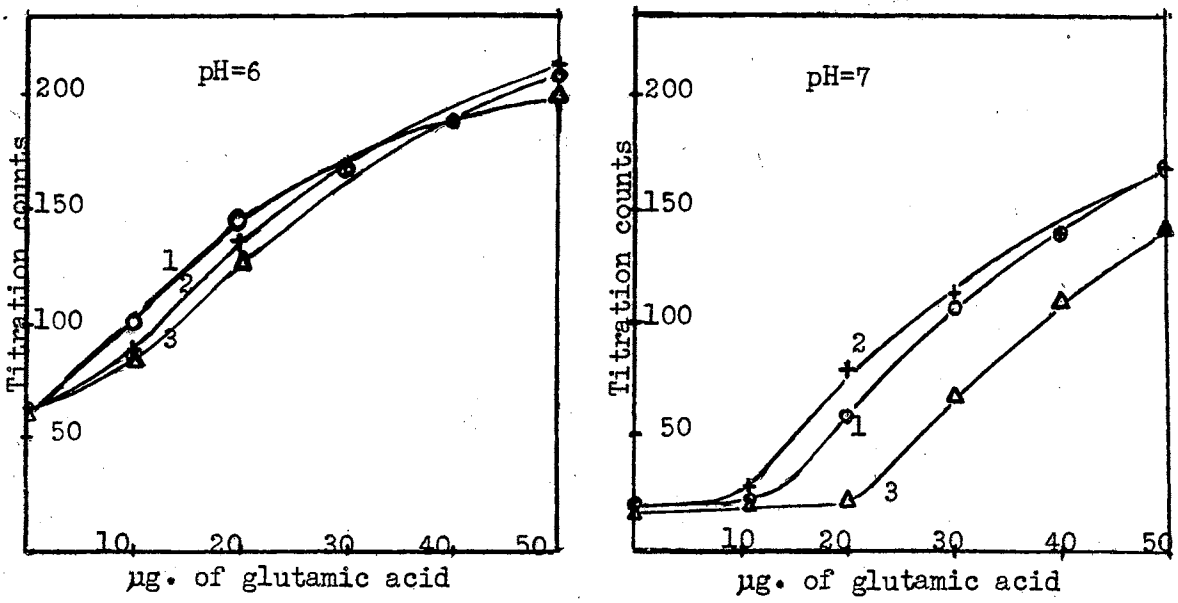


Fig. 2 a and b. (1) Low arginine, (2) low aspartic acid, (3) low leucine. Glycine, serine and tyrosine at 50 per cent of the normal H-S concentration in all tubes.

Figures 3 and 4

Response of L. arabinosus to glutamic acid under various conditions.

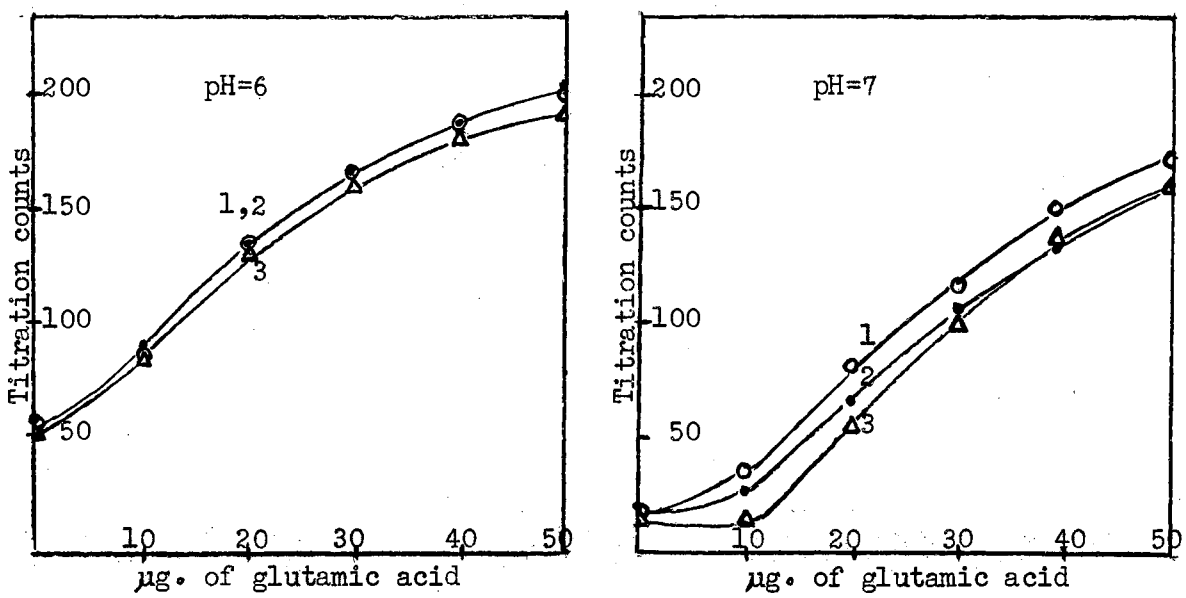


Fig. 3 a and b. (1) low leucine and aspartic acid, (2) low arginine and aspartic acid, (3) low arginine and leucine. Glycine, serine and tyrosine at 50 per cent of the normal H-S concentration in all tubes.

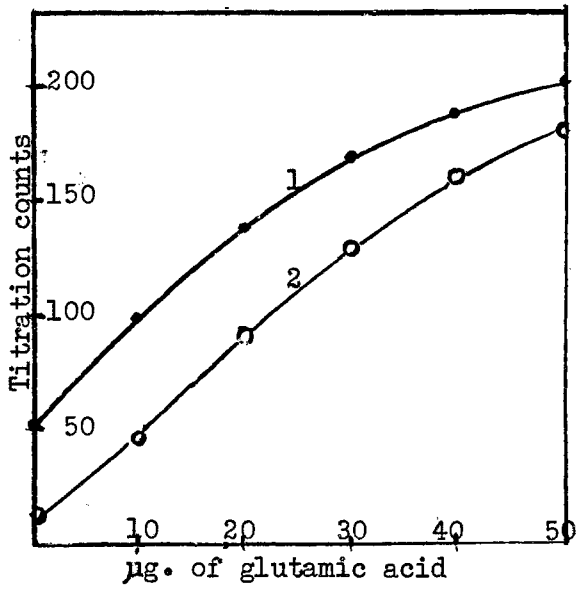


Fig. 4. (1) pH 6 and (2) pH 7. Arginine, aspartic acid and leucine at 10 per cent and glycine, serine and tyrosine at 50 per cent of the normal H-S concentration.

B. Inhibition of Utilization of Amino Acids by Lowering the Level of Other Amino Acids in the Medium.

The only instance of this kind of amino acid interrelationship previously known and studied was the arginine-proline interdependence reported by Sirny et al. (35, 40).

With the discovery of the great number of other similar interdependences in the previous survey it was found desirable to see if the original observations were specific for the arginine-proline interdependence, or if they would hold generally for all similar interrelationships. This part of the present research was, therefore, designed to investigate the effect of pH, of partial protein hydrolysates, and, ultimately, of synthetic dipeptides on several of these amino acid interdependences.

Experimental:

The first step was to confirm by re-examination the reversible interdependences for Leuconostoc mesenteroides at pH 6 and 7. This repetition was accomplished in accordance with the general procedures under conditions as uniform as possible. The experiments were set up to make possible comparison of the response to limiting amounts of amino acid A in the presence of normal and 1/10 of the normal amount of the modifying amino acid B, and the response to limiting B in the presence of normal and 1/10 of the normal amount of A.

The next step was to investigate the effect of partial protein hydrolysates on some of the most striking interdependences for Leuconostoc mesenteroides. The hydrolysates were prepared by hydrolyzing 1 gm. of casein with 40 ml. of 3N HCl in the autoclave at 120° C. For the partial hydrolysis the autoclaving time was 1 hour, and for the complete

hydrolysis, 15 hours. After rapid cooling, the pH of the hydrolysates was adjusted to approximately 4, and they were made up to 250 ml. volume. After proper dilution, based on the amino acid percentage in crude protein (obtained from the data of Steele et al. (65)) these hydrolysates were used for standards in the assays along with the particular amino acid involved.

The following interdependences were investigated using the concentrations given in Table I, (Part I).

Arginine - Glycine	Glycine - Arginine
Arginine - Serine	Serine - Arginine
Arginine - Threonine	Threonine - Arginine
Proline - Histidine	Histidine - Proline
Proline - Threonine	Threonine - Proline

Consideration was given to the increased concentration of the modifying amino acid brought about by addition of the hydrolysates. The increase was in some instances as much as 50%, and this was considered very carefully in evaluating the results.

The last step was the investigation of the effects of different glycine containing peptides on the glycine-arginine interdependence in Leuconostoc mesenteroides. The glycine-arginine interdependence was selected because it is a very striking one, and because several glycine peptides are readily available.

The experiments were simply set up to compare the effect of high and low arginine on the utilization of glycine supplied as the following compounds:

Glycine	10 ug./ml.
Glycyl-glycine	8.8 ug./ml.
Glycyl-alanine	19.5 ug./ml.
Alanyl-glycine	19.5 ug./ml.
Glycyl-leucine	25.07 ug./ml.
Leucyl-glycine	25.07 ug./ml.

The above concentrations are all calculated to be equivalent to 10 ug. glycine per ml.

Results:

The repeated experiments on the reversible interdependences served to confirm most of the results from the survey. There were, however, some deviations, mainly in the magnitude of interdependence for some amino acids. This indicates that there are other variables which are not yet completely controlled. In general, the effect of lowering the pH to 6 proved to be a decrease in the magnitude of the interdependence, i.e., a more optimal response to the limiting amino acid was obtained even in the medium containing only low amounts of the modifying amino acid.

The growth curves with the hydrolysates in the arginine-glycine and glycine-arginine interdependences are shown in Figs. 5 and 6. Not only did the amino acids supplied in the form of partial hydrolysates nearly completely overcome the interdependences, but they also gave increased growth response on the medium containing "high" arginine. A similar stimulation of growth was not observed with the 15 hour hydrolysates which contain the limiting amino acid at exactly the same concentration as the 1 hour hydrolysates. The graphs also show that lowering pH from 7 to 6 decreases the magnitude of the interdependences and therefore, also, the apparent stimulatory effect of the partial hydrolysates.

When dipeptides were supplied as the source of glycine in the glycine-arginine interdependence, the growth curves were very similar to those obtained with the incomplete hydrolysates, giving a markedly increased growth response as compared to the free amino acid. This indicates that the stimulatory effect of the incomplete hydrolysates is due

to the presence of peptide-bound amino acids which evidently are more readily utilized than the free amino acids by this organism under these conditions. The actual results from the experiments involving synthetic peptides are shown in Fig. 7. Glycyl-glycine is the least effective dipeptide in overcoming the interdependence but it is still utilized much more efficiently than glycine itself, both with high and low arginine in the medium. The other peptides completely overcome the interdependence, some of them being utilized, even more efficiently in the medium containing low arginine than is free glycine in the medium containing high arginine.

The dipeptides were assayed for glycine in a separate experiment. Some deviations from the theoretical values were encountered, but were not great enough to explain the effect of the peptides on the interdependence.

In agreement with the data from the present study as well as data obtained in the literature, the following seems to be true for the relative utilization efficiency of free amino acids and peptide bound amino acids for the lactic acid bacteria:

Under optimal growth conditions the amino acids are presumably utilized with 100 per cent efficiency, and under these conditions the peptide bound amino acids are used less efficiently than the free amino acids. The degree to which the peptide bound amino acid is used as compared to the free amino acid has been reported to vary between 0 and 100 per cent.

If the growth conditions become suboptimal for the organism, the free amino acids are utilized correspondingly less efficiently. In this work

suboptimal conditions presumably were produced by keeping the pH high or by removing or altering the concentration of some stimulating component of the medium. Whatever the mechanism by which these inhibiting conditions are brought about may be, they apparently affect only the utilization of the free amino acid, and allow the peptide bound amino acid to be used with the same efficiency as it was under optimal conditions. Comparatively, thus, it will appear that the peptide stimulates growth, and the magnitude of stimulation will be dependent on the magnitude of the inhibition exerted upon the utilization of the free amino acid.

This explanation is in agreement with the few observations of peptide stimulation, which have been reported and discussed in the literature (40).

Summary and Conclusions:

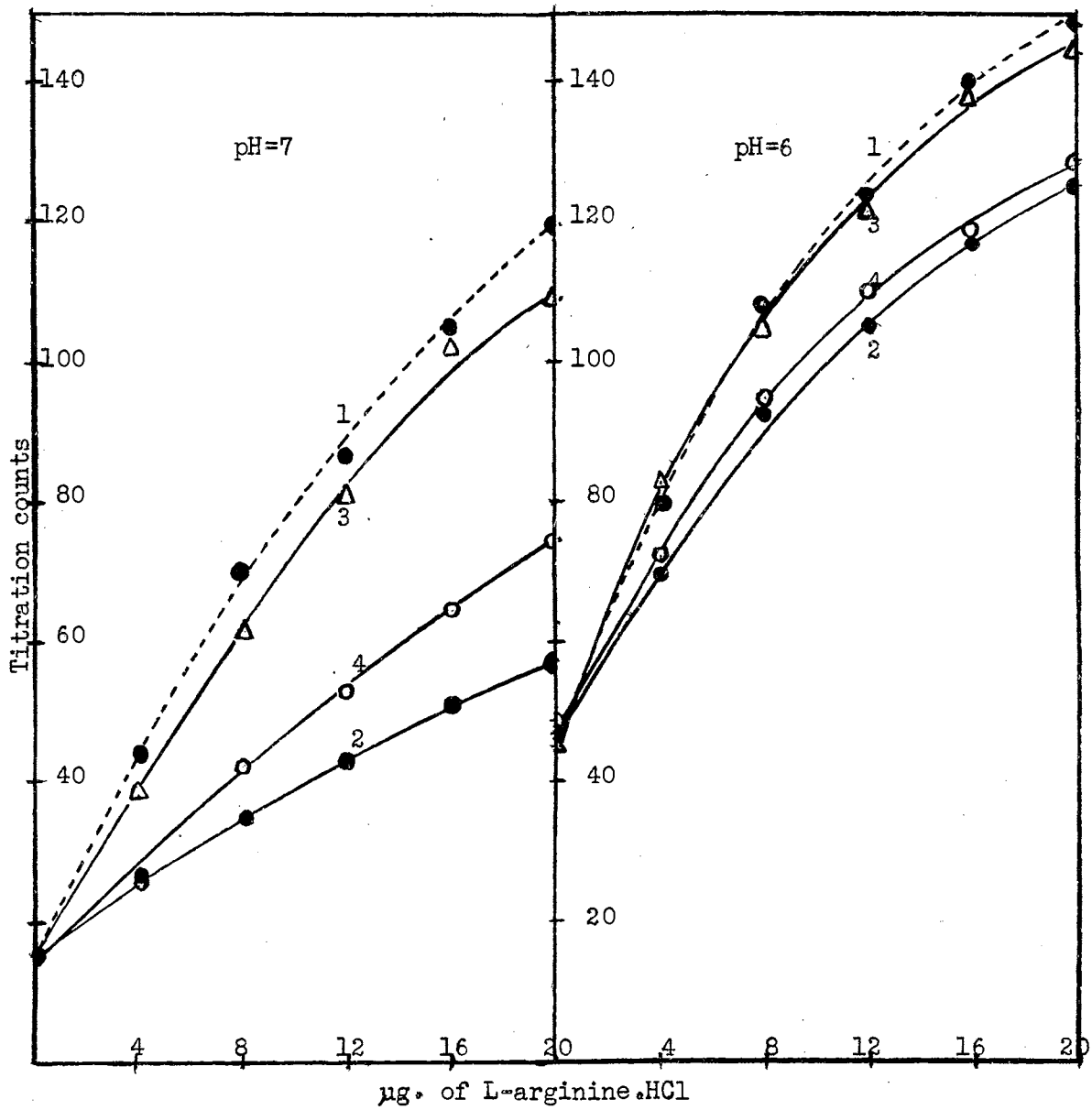
Several cases of amino acid interdependences for Leuconostoc mesenteroides have been repeated and found to be true and reproducable phenomena. The fact that lowered pH and incomplete protein hydrolysates are capable of reducing the requirement for high levels of a modifying amino acid in the medium for optimal utilization of the limiting amino acid, which was reported in the original arginine-proline interdependence, has been verified and shown to be a general characteristic for all the interdependences investigated.

Supplying the limiting amino acid in the form of synthetic dipeptides also eliminated the requirement for a high level of the modifying amino acid. It has been concluded that the inhibitory conditions produced by the reduced concentration of the modifying amino acid only affect the utilization of the free amino acid, leaving the utilization of the amino

acid of the dipeptide uninhibited. Under these inhibited conditions, therefore, the peptide is more readily utilized than the free amino acid and thus appears to be stimulatory to growth.

Figure 5

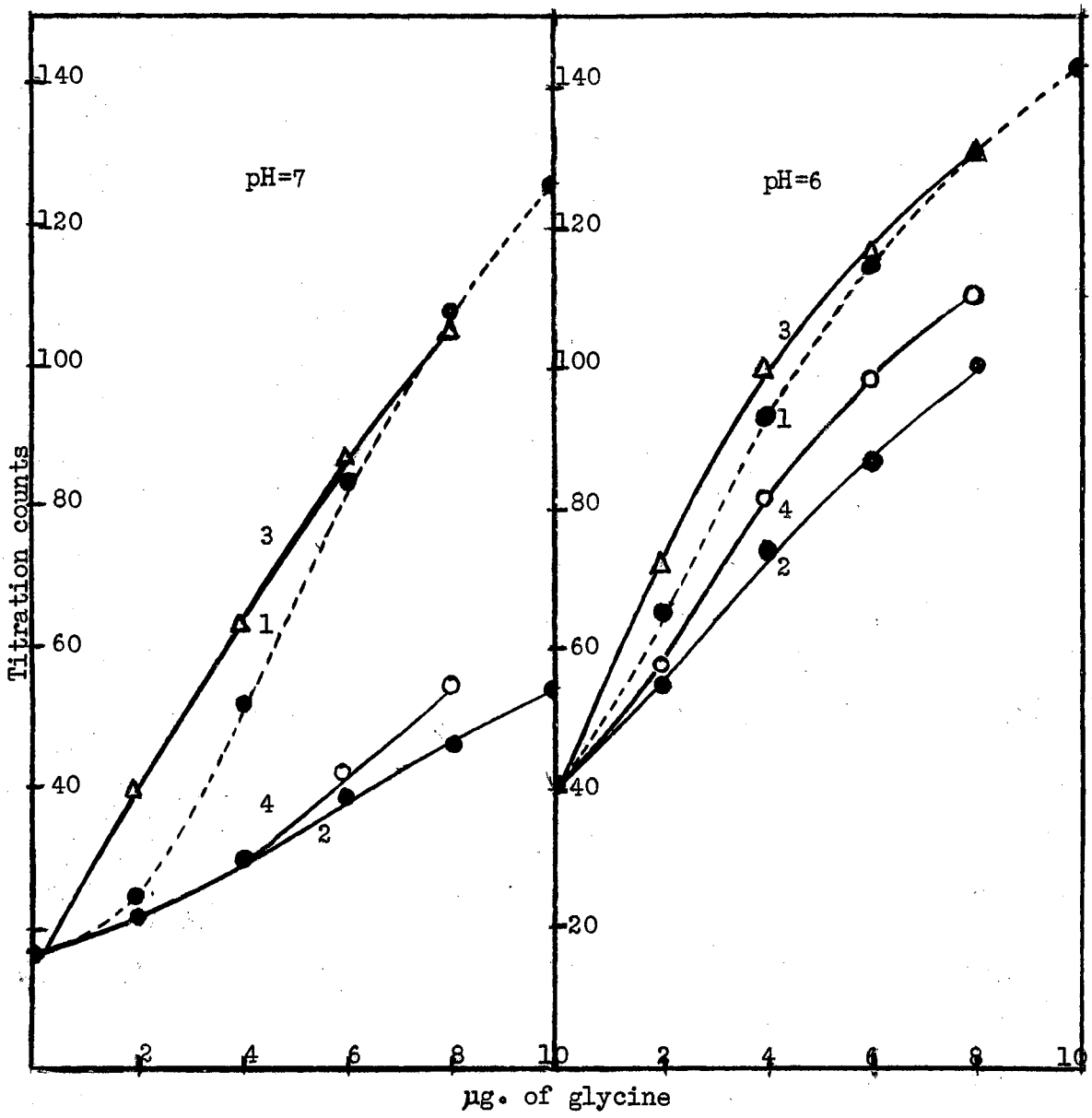
The effect of partial hydrolysates on the arginine-glycine inter-dependence with Leuc. mesenteroides.



(1) Response to arginine in the presence of "high" glycine.
(2) Response to arginine in the presence of "low" glycine.
(3) Response to arginine from partial hydrolysates in the presence of "low" glycine.
(4) Response to arginine from complete hydrolysates in the presence of "low" glycine.

Figure 6

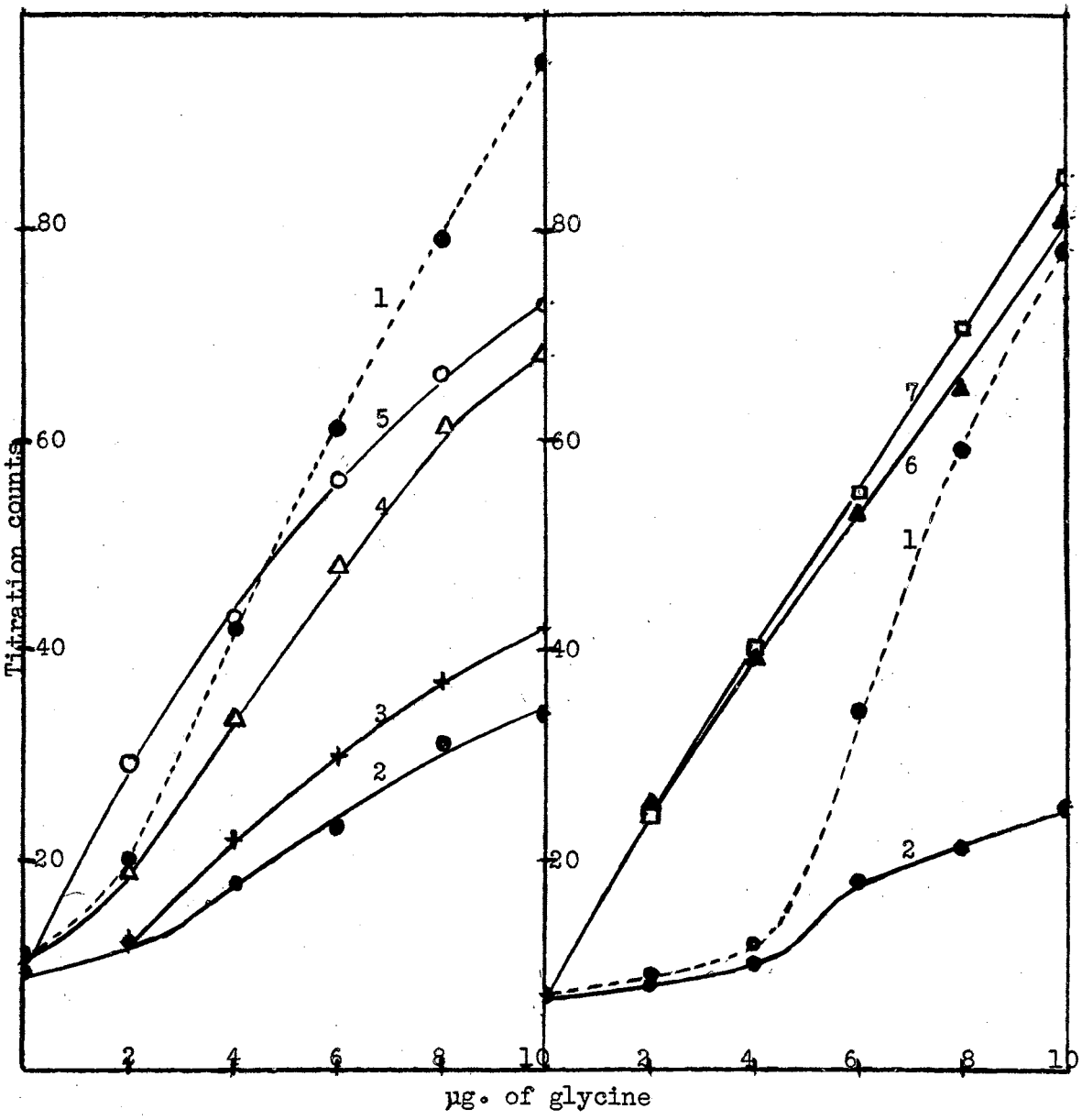
The effect of partial hydrolysates on the glycine-arginine interdependence with Leuc. mesenteroides.



(1) Response to glycine in the presence of "high" arginine.
(2) Response to glycine in the presence of "low" arginine.
(3) Response to glycine from partial hydrolysates in the presence of "low" arginine.
(4) Response to glycine from complete hydrolysates in the presence of "low" arginine.

Figure 7

The effect of several glycine dipeptides on the glycine-arginine interdependence with Leuc. mesenteroides.



(1) Response to glycine in the presence of "high" arginine.
(2) Response to glycine, (3) to glycyl-glycine, (4) to glycyl-alanine, (5) to alanyl-glycine, (6) to leucyl-glycine and (7) to glycyl-leucine, all in the presence of "low" arginine. pH 7.

C. Inhibition of Synthesis of Amino Acids by Other Amino Acids in the Medium.

Perhaps the most surprising effect revealed in the previous survey was the inhibition of synthesis of certain amino acids by other amino acids at the levels customarily used in the medium. This effect becomes manifest as a high blank in the growth curve (absence of limiting amino acid) when the concentration of the inhibiting amino acid was lowered to one tenth of the normal concentration.

The difference in the amino acid requirement of the different species of the lactic acid bacteria is undoubtedly due to difference in their capacity to synthesize amino acids, and the literature contains several examples of this. Apparently, however, it has not been recognized that decreasing or even eliminating one amino acid in the medium can completely eliminate the requirement for another amino acid for the organism involved. This is what shall be discussed in the following, in relation to the alanine inhibition of serine synthesis for Leuconostoc mesenteroides.

This case of inhibition was selected for further study for several reasons. Firstly it appeared as a very pronounced interrelationship in the earlier studies, and secondly there has been quite a lot of work done with regard to the biosynthesis of serine. Greenberg and associates (72) showed by tracer techniques that glycine is converted to serine in vitro in the presence of liver homogenate, and that the required formate group originates from the alpha carbon of glycine (73). The same was demonstrated by Sakami (74) by somewhat different techniques. Sakami also fed rats C^{14} tagged formate and glycine with the carboxyl group tagged with C^{13}, and showed that a large amount of serine isolated from

liver proteins contained C^{13} in the carboxyl group and C^{14} in the beta-carbon position (75). The formation of glycine and formic acid from serine has been demonstrated by Shemin (76) in rats and guinea pigs.

In 1950 Lascelles and Woods (77) reported synthesis of serine by Leuconostoc mesenteroides. They found that in a medium containing 1 mg. of alanine per 2 ml. assay volume glycine plus pyridoxal could partly replace the serine requirement known to exist for this organism. If para-amino benzoic acid was also added, the serine requirement was almost entirely eliminated. Serine synthesis in other organisms has also been reported, as mentioned earlier. Streptococcus faecalis can synthesize serine when folic acid is supplied, and both Streptococcus faecalis and Lactobacillus arabinosus can grow without serine when vitamin B_6 is supplied (17).

Experimental:

The experiments were set up to study the different factors involved in the inhibition of serine synthesis. The ultimate aim was to determine the mechanism or mechanisms by which the synthesis of serine by Leuconostoc mesenteroides is inhibited by alanine.

The experimental procedures were relatively simple, the different factors that were to be studied were added as standards to the assay, and their effect tested in serine free media. The general experimental conditions were kept as uniform as possible, to allow reproduction of results and quantitative comparison between assays. The cells of Leuconostoc mesenteroides used for the assays were grown on the normal liquid medium (Appendix B) which does contain serine.

Results:

The original data from the survey are shown in Fig. 8a and b. The continued investigation brought forth the following observations:

Serine synthesis is inhibited by DL-alanine, and L-alanine is the effective inhibitor, while the effect of D-alanine is little or none. Near maximal growth in the absence of serine is obtained when alanine is completely omitted from the medium, and alanine is obviously not required by this organism. With the normal level of alanine which is 2000 ug./tube, the serine synthesis is only partly inhibited, which is shown by relatively high and irregular blanks. Complete inhibition is accomplished with 4000 ug. DL-alanine or 2000 ug. L-alanine (Fig. 9a).

Since it has been shown that glycine is essential for serine synthesis, and the survey showed that alanine does interfere quite markedly with the glycine utilization for the organism at hand, there is some clue as to the mechanism of the alanine inhibition.

The inhibition by cystine is less marked, but still significant. There is, however, one major difference in that while alanine is not required by the organism, cystine definitely is, and the optimal growth conditions with respect to cystine concentration is obtained at a level of approximately 40 ug./tube (Fig. 9b). The normal level of 200 ug./tube of cystine does not give optimal growth (serine synthesis), but in order to prevent the involvement of too many variables, this level was nevertheless maintained for the further studies. Increasing the cystine level above the normal does not result in complete inhibition of serine synthesis in the absence of alanine. It is important to note that neither alanine nor cystine interfers with serine utilization.

Since glycine seems to be one of the limiting factors in the synthesis of serine, it was a logical step to investigate the effect of arginine, in view of the very marked glycine-arginine interdependence which has already been studied. As expected, arginine is definitely involved. When the arginine level is lowered to one tenth of the normal level, the serine synthesis is completely blocked, even in the absence of alanine. If increased above the normal level, arginine gives some stimulating effect (Fig. 10).

Serine synthesis is completely eliminated if the B_6 vitamins are omitted from the medium. In promoting serine synthesis, pyridoxal is the most effective with pyridoxamine next. Pyridoxine apparently has no effect (Fig. 11). One separate experiment revealed that pyridoxal phosphate and pyridoxamine phosphate are still more effective in promoting serine synthesis. The data from this experiment even indicate that the phosphorylated compounds can overcome the alanine inhibition.

Glycine has already been mentioned as being required for the serine synthesis, and maintaining normal levels of arginine, cystine and pyridoxal, the alanine inhibition is overcome by increased amounts of glycine above the normal level of 200 ug./tube. Glycyl-glycine is approximately twice as effective as glycine itself in overcoming the inhibition (Fig. 12).

Folinic acid also overcomes the alanine inhibition under otherwise normal conditions. This was first found using Reticulogen (Lilly) and later confirmed by supplying the vitamin in the form of the synthetic Ca-Leucovorin[4] (Fig. 13). The growth of the organism, and thus the synthesis of serine, is still dependent on the amount of glycine present,

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[4]Generously supplied by the Lederle Laboratories.

however. No matter how much of the vitamin is supplied, glycine remains the limiting factor. A comparison of the amounts of glycine required to give a certain arbitrarily chosen amount of growth in an inhibited system with and without folinic acid resulted in the ratio 1:2.08. This is the average from several assays and several levels of growth, (total 36), and it means that only one half as much glycine is required when the vitamin is supplied.

From the survey it was seen that when the alanine in the medium is lowered to 1/10 of the normal level, the response of Leuc. mesenteroides to glycine is much better. It seems likely, therefore, that the alanine interference in the serine synthesis may be the result of inhibition of glycine utilization. Similarly, arginine also seems to be involved in the serine synthesis by virtue of its influence on the glycine utilization. The probable mechanism of the effect of these two amino acids will be discussed later.

The effect of leucovorin in overcoming the alanine inhibition is to spare glycine in some way. The following mechanism might be proposed:

Under normal noninhibited conditions the synthesis of serine could conceivably follow the pathway:

$$2 \text{ glycine} \xrightarrow{\text{pyridoxal}} \text{serine}$$

If this reaction were in some way impaired by high amounts of alanine or low amounts of arginine, possibly through less efficient utilization of glycine, serine might be synthesized through another pathway requiring less glycine:

$$\text{glycine} + (\text{CHO}) \xrightarrow{\text{folinic acid}} \text{serine}$$

This pathway would presumably only be used when folinic acid is supplied. The nature of the one-carbon fragment which would necessarily be involved

in this second reaction is not known. Addition of formate to the medium did not have any effect under the present conditions. This was surprising since it is known that folinic acid is involved in one-carbon metabolism and that it itself posesses a formyl group as part of the molecule. It is possible that the one-carbon fragment might need to be in a form more active than free formate. A medium as complex as the one used here undoubtedly contains several forms of formate-like compounds, which might react with glycine to give serine.

The pH of the medium was also found to affect the synthesis of serine. If the pH is lowered, the alanine inhibition decreases and the serine synthesis, therefore, procedes more readily (Fig. 14). The effect of pH is very similar to that already pointed out for the other amino acid interrelationships above.

Summary and Conclusions:

Several ways of blocking the synthesis of serine from glycine in Leuconostoc mesenteroides have been shown. Four mg. DL-alanine or 2 mg. L-alanine per tube completely inhibit the synthesis under the normal conditions. The inhibition can be overcome by decreasing the amount of alanine, or increasing the amount of glycine. More efficient utilization of glycine, as accomplished with glycyl-glycine or glycine + leucovorin also overcomes the inhibition.

The results should have several practical applications. Firstly, an improved assay of serine can be obtained by using 4000 ug. DL-alanine per tube instead of the normal level of 2000 ug. Under normal conditions the serine assay with Leuconostoc mesenteroides is obscured by high and inconsistant blanks. This can now be prevented by the use of the high

amounts of alanine, and since alanine does not appear to interfere with serine utilization in any way, a very good assay of serine is secured.

Secondly, the results may be helpful in revealing more information as to the mode of action of folinic acid. Relatively little is known about this vitamin and its functions, and this seems to be a simple and convenient system by which more data can be obtained.

Figures 8 and 9

Different factors affecting serine synthesis in Leuc. mesenteroides.

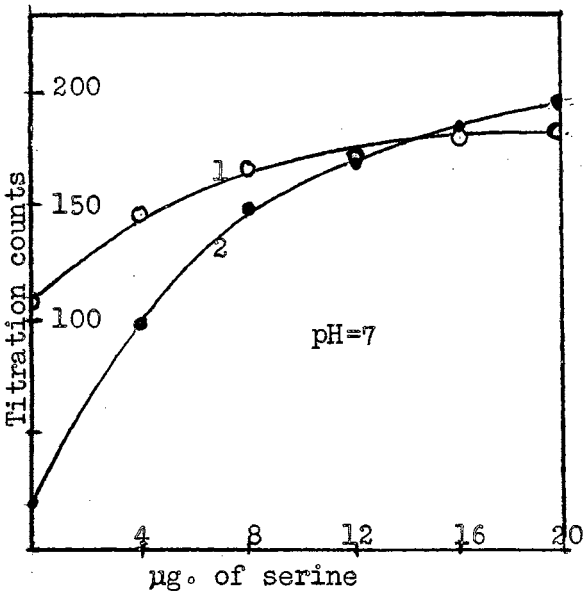

Fig. 8a. Original survey results on the effect of (1) "low" alanine and (2) "high" alanine.

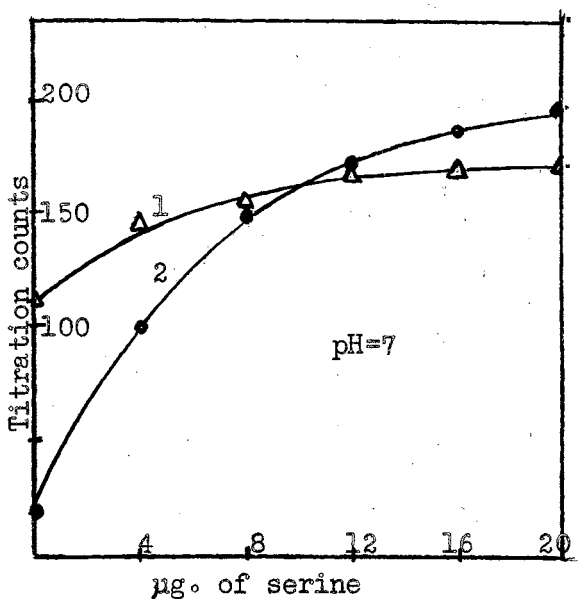

Fig. 8b. Original survey results on the effect of (1) "low" cystine and (2) "high" cystine.

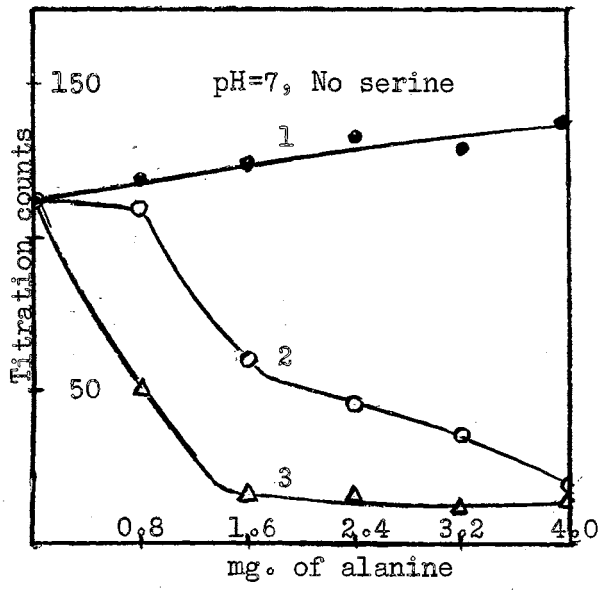

Fig. 9a. Effect of (1) D-alanine, (2) DL-alanine and (3) L-alanine.

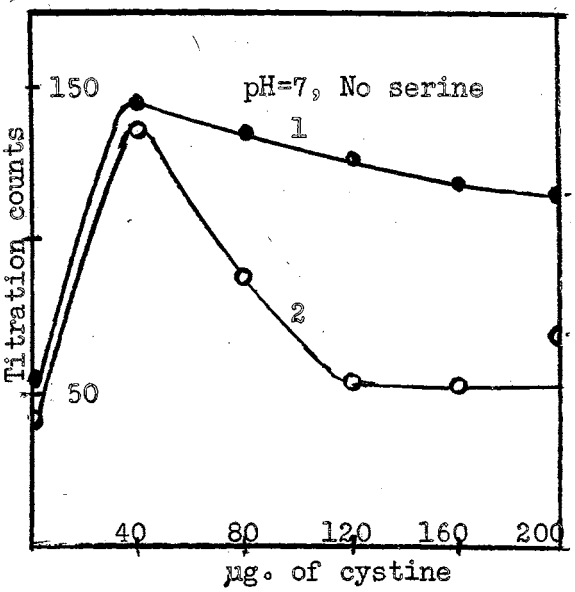

Fig. 9b. Effect of cystine in the presence of (1) 1 mg. and (2) 2 mg. DL-alanine.

Figures 10 and 11

Different factors affecting serine synthesis in Leuc. mesenteroides.

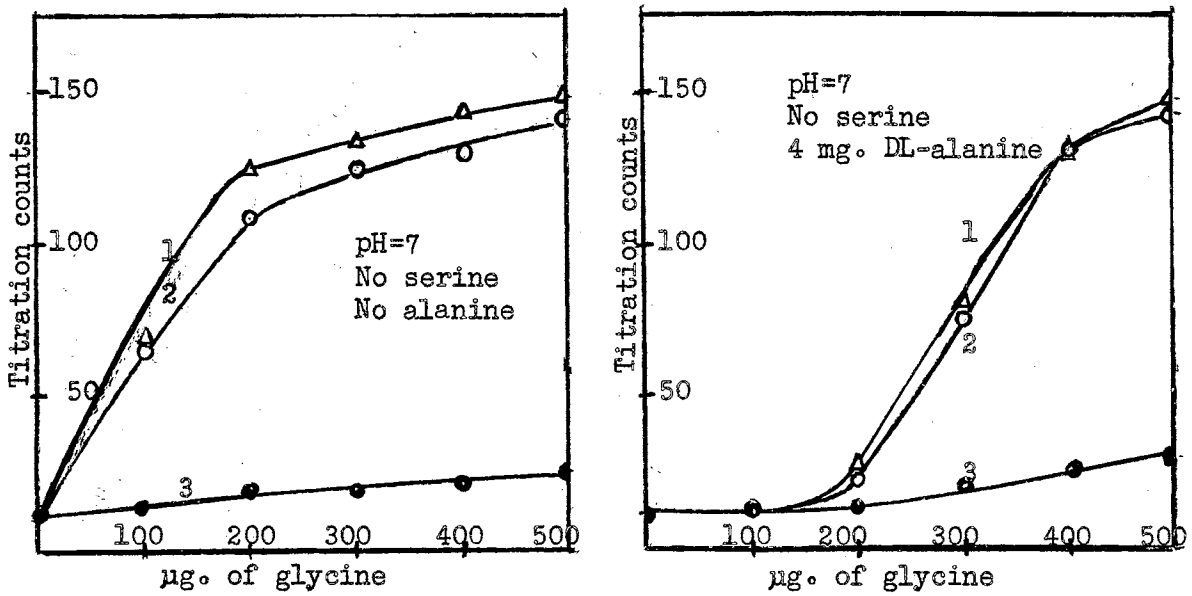

Fig. 10. The effect of (1) 2000 µg., (2) 400 µg. and (3) 40 µg. of arginine on the serine synthesis in response to glycine.

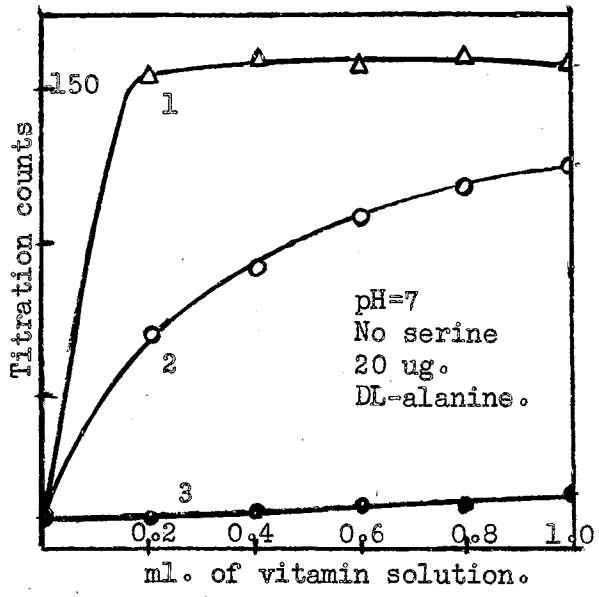

Fig. 11. The effect of different forms of vitamin B_6 on serine synthesis. (1) Pyridoxal (4 µg./ml.), (2) Pyridoxamine (4 µg./ml.) and (3) Pyridoxine (40 µg./ml.)

Figures 12, 13 and 14

Different factors affecting serine synthesis in *Leuc. mesenteroides*.

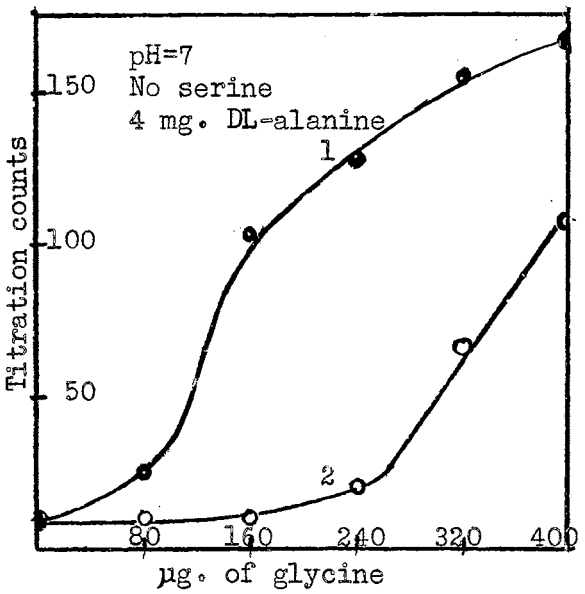

Fig. 12. The effect of (1) glycyl-glycine as compared to (2) glycine on serine synthesis.

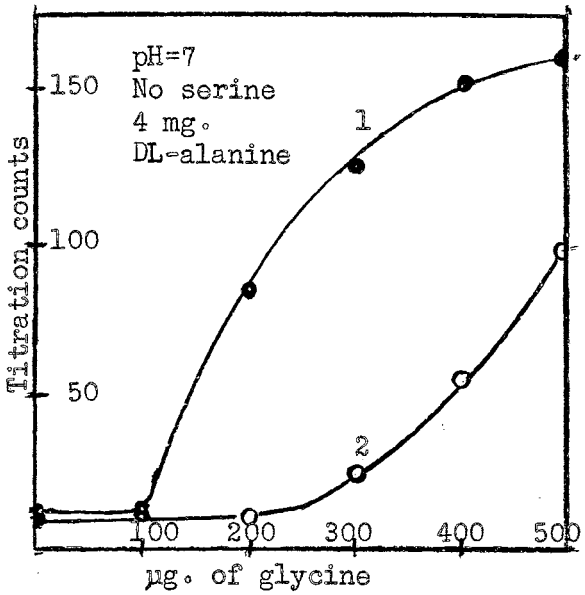

Fig. 13. Response to glycine in (1) presence and (2) absence of folinic acid (Leucovorin).

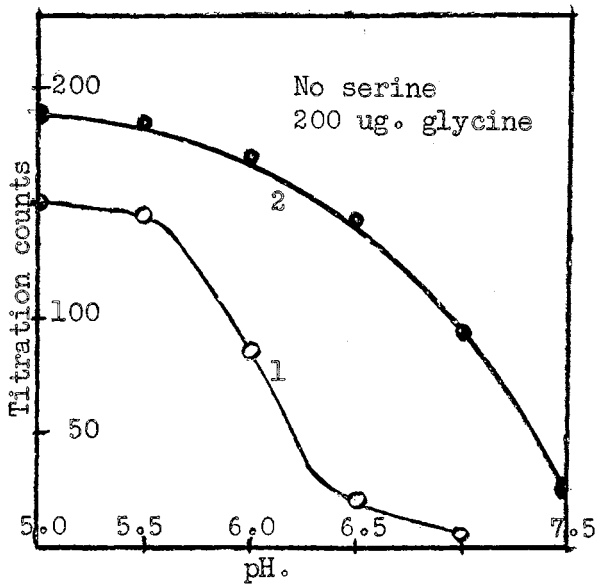

Fig. 14. The effect of pH on serine synthesis (1) in the presence of 4 mg. DL-alanine and (2) in the absence of alanine.

PART III

PRELIMINARY INVESTIGATIONS OF AMINO ACID ASSIMILATION

The great number of amino acid interrelationships revealed in the previous survey are too general in nature to be explained individually in terms of specific interrelationships. The similar effects of pH, peptides and also, as is found in other phases of this work, ion concentration on the different kinds of interrelationships further indicate that a more general phenomenon is involved as the cause of the many interrelationships. It is the elucidation of such a phenomenon that must be sought.

In looking for a step in the amino acid metabolism in the lactic acid bacteria which might be the same regardless of the character of the amino acid, the first and logical place to investigate is the passage of the amino acid through the cell wall, the assimilation of the amino acid. The mechanisms by which all the different 18 amino acids are assimilated are hardly exactly identical, but undoubtedly more so than any of the intracellular processes by which the amino acids are utilized.

A survey of the literature showed that this may indeed be the right place to look for the explanation of some of these interrelationships. Gale and coworkers in England have demonstrated that gram positive bacteria not only take up amino acids, but even accumulate them within the cells to a concentration up to 400 times that in the external medium. Some amino acids are taken up by simple diffusion, while others require a source of energy, e.g., glucose, large amounts of ATP or arginine, the

latter being shown to promote uptake of glutamic acid by Streptococcus faecalis (a glutamic acid-arginine interdependence was found for this organism in the previous survey). Their experiments were performed in a medium containing only salts, the amino acid investigated and glucose. This medium does not promote growth, so the cells were in a resting state. The experimental procedures and the results from several far-reaching studies are reported in a series of 16 papers (78 - 93) and a review (94).

Other workers have studied several phases of amino acid assimilation in other cells. Christensen and coworkers have worked with concentrative uptake of amino acids by erythrocytes, reticulocytes and free neoplasm cells (95 - 99). The interesting part of their work is that they have demonstrated a definite effect of other amino acids on the uptake of one amino acid, in that some of them stimulate the uptake, while others inhibit it. In the case of the free neoplasm cells, they demonstrated a mutual inhibition of uptake for glutamic acid and aspartic acid, and also that alanine is the most effective inhibitor for glycine uptake, while arginine stimulates the uptake of this amino acid. While it was realized that lactic acid bacteria might not be expected to behave exactly like free cells of the Ehrlich mouse ascites tumor, it was deemed worth while to try to demonstrate similar relationships for the lactic acid bacteria, especially with reference to the specific cases of amino acid interrelationships studied above.

Experimental:

The buffer solution, which was used for all the following experiments is that used by Gale in his work. Its composition is given in Appendix C and it will be referred to as "Gale's solution."

Cells for the experiments were grown on a normal Henderson-Snell medium deficient in the amino acid to be investigated, usually at a volume of 1000 ml. After 24 - 30 hours growth the cells were harvested by centrifuging, washed once with saline and then made up to a concentrated suspension with Gale's solution (previously adjusted to pH 7). One ml. of this suspension was taken out and dried in vacuum desiccator so that the dry weight of cells in the suspension could be determined.

The amino acid to be investigated was dissolved in Gale's solution at a concentration which was five times that desired for the actual run. Other additions, such as glucose or other amino acids, were weighed in simultaneously so that the original solution would contain all the components of the final medium, all five times more concentrated than in the final medium. Then 1 ml. of this concentrated solution was placed into each of two tubes. To one of these tubes (to be termed the blank) was added 4 ml. of Gale's solution, and to the other 4 ml. of the previously prepared cell suspension. The tubes were shaken up and incubated for 1 - 2 hours at 37° C. After incubation they were centrifuged, and the supernatants were quantitatively diluted to the required concentration for the amino acid assay. The assays were set up with these diluted solutions as standard curves, and comparisons were then made between the blank and the cell treated sample as to the content of the amino acid in question. If the assay revealed any difference between the blank and the solution which had been incubated with cells, this difference was assumed to be due to the cells, since that was the only variable between the two solutions.

In calculating the results as micrograms of amino acid taken up, the blank was plotted as the standard curve in each case, and the amount of

amino acid left after uptake in the cell treated sample was obtained from this curve in per cent. Knowing the per cent of amino acid removed from the medium, the original concentration of the amino acid and the number of milligrams (dry weight) of cells present, the uptake could be calculated as micrograms of the amino acid taken up per milligram of cells.

Several experiments were conducted on glycine uptake by Leuc. mesenteroides. The concentrations used were 5 micromoles of glycine (or glycine as glycyl-glycine), and glucose and 50 micromoles of alanine and arginine whenever these were added. The concentrations of vitamins when used were arbitrarily chosen levels in excess of the organism's requirement.

The glycine assay with Leuc. mesenteroides was performed in a medium which, in accordance with observations from the earlier survey, contained alanine at 1/2 normal level, and at pH 6.

In some experiments the same solutions were also assayed for alanine, using the normal Henderson-Snell medium with reticulogen added and with Leuc. citrovorum as the assay organism.

In order to investigate other conditions than those applying to the serine synthesis problem, a study of glutamic acid uptake in the absence and presence of aspartic acid and asparagine was made. The concentrations used were 10 micromoles of glutamic acid and glucose and 100 micromoles of aspartic and asparagine. Cells of L. arabinosus grown on a low glutamic acid medium were used, and glutamic acid was assayed with that organism under the conditions recommended earlier in this work (page 32) at pH 6.

Results:

The results from the glycine and alanine uptake experiments are given in Table VI and Table VII respectively, and the results from the glutamic acid uptake experiment in Table VIII. Although the results are yet far from conclusive, they are considered indicative that further work may yield a possible explanation of the complex amino acid interrelationships for the lactic acid bacteria.

Especially in the case of glycine uptake, the results are not very clear and informative, mainly because it has not been possible to accomplish any uptake of glycine under the conditions used. The fact, however, that in all trials glycyl-glycine is taken up under the very same conditions should give conclusive evidence for the theory that the peptide is taken up more readily than the amino acid itself. Less conclusive, but still notable are the results with the alanine inhibition of the uptake of glycyl-glycine, since a consistant, however small, decrease in uptake is observed when alanine is present. Correlating these data with those from the alanine uptake indicates that alanine may inhibit glycine uptake because it is more readily taken up by the cells and thus causes an increase in the internal concentration of amino acids. This in turn causes a gradient which is unfavorable to glycine uptake. The fact that L-alanine apparently is taken up much more readily than D-alanine may explain why L-alanine is the effective inhibitor. Moreover, it is even possible that D-alanine, rather than being taken up, has the effect of withdrawing alanine (presumably L-alanine) from the cells. This again may explain the slight stimulatory effect of D-alanine on the serine synthesis in Leuconostoc mesenteroides.

It was not possible to demonstrate any arginine effect on the uptake of glycyl-glycine, and this probably is due to the fact that, as already shown, the effect of all the amino acids are much less pronounced when the limiting amino acid is supplied in the form of a dipeptide.

In the classical case of aspartic acid inhibition of glutamic acid utilization, the results from only one trial clearly indicate that the inhibition is due to blocking of the glutamic acid uptake. Asparagine, which can replace aspartic acid for growth of L. arabinosus, blocks the glutamic acid uptake to a much lesser degree than does aspartic acid itself. According to the present data aspartic acid apparently completely prevents the uptake of glutamic acid. These observations definitely are in agreement with data presented by earlier workers as to the glutamic acid-aspartic acid competitive inhibition.

The techniques which lead to the varying results in this part of the work can undoubtedly be greatly improved. It seems to be a question of formulating more standard conditions, which should be simple in view of the relatively small number of variables in this type of study. The main problem at present seems to be the growing of the cells used in the uptake experiments. Gale and his coworkers have shown in their work that the age of the culture is very important, and experience from the few previous experiments clearly has shown that the composition of the medium on which the cells are grown must be controlled with similar care. This is especially true in the glycine uptake studies, in which the levels of both glycine, alanine and serine are all of importance.

Summary and conclusions:

A method has been described by which ordinary amino acid assay methods

can be conveniently used to determine amino acid assimilation in different lactic acid bacteria. The limitations have been pointed out together with the suggestion that these limitations are not impossible to control.

The results are not conclusive, but they are in agreement with the proposed hypothesis that the amino acid interrelationships discussed in the previous parts of this work are results of the effect of certain amino acids on the uptake of the limiting acid.

By use of improved methods, studies of this kind seem very promising in giving the answers to several of the unexplained questions in the field of microbial nutrition and metabolism.

Table VI. Glycine uptake by Leuconostoc mesenteroides.

CONDITION	TRIALS	UPTAKE OF GLYCINE UG/MG CELLS I	II	III	IV	AVERAGE	NO. OF TRIALS
Glycine + glucose pH 7			-1.0,	1.5,	-0.1	0	3
Glycine + glucose + alanine pH 7				-1.95			1
Glycyl-glycine + glucose pH 6			31.25				1
Glycyl-glycine + glucose pH 7		11.6,	15.0,	14.6,	12.6	13.5	4
Glycyl-glycine + glucose + DL-alanine pH 7				8.1,	8.7	8.4	2
Glycyl-glycine + glucose + L-alanine pH 7				11.0,	6.9	9.0	2
Glycyl-glycine + glucose + D-alanine pH 7				10.2,	10.0	10.0	2
Glycyl-glycine + glucose + DL-alanine + folinic acid pH 7				9.7			1

Table VII. Alanine uptake by Leuconostoc mesenteroides.

CONDITION	TRIAL	UPTAKE OF ALANINE UG/MG CELLS III	IV	AVERAGE
DL-alanine + glycyl-glycine + glucose	pH 7	2.3	-5.7	-1.7
D-alanine + glycyl-glycine + glucose	pH 7	1.6	-25.5	-12.0
L-alanine + glycyl-glycine + glucose	pH 7	57.0	48.0	52.5

Table VIII. Glutamic acid uptake by *Lactobacillus arabinosus*.

CONDITION	UPTAKE OF GLUTAMIC ACID UG/MG CELLS
Glutamic acid + glucose	35.8
Glutamic acid + glucose + aspartic acid	-1.13
Glutamic acid + glucose + asparagine	26.5

GENERAL DISCUSSION

The difficulties encountered in finding a simple way to explain the various results in this work have undoubtedly been evident through all the previous parts. It has been definitely established that the four groups of effects resulting from lowering the concentration of one of the nonlimiting amino acids in the medium are true and reproducible phenomena, and it seems likely that they should all be expressions for one property of bacterial cells.

The literature contains several reports concerning amino acid interrelationships, and it seems to be presumed in each case that the mechanism of the relationship depends on specific intracellular, enzyme chemical reactions. The more of these interrelationships are revealed and the more general the phenomena are found to be, the more untenable this explanation becomes. In spite of the somewhat limited data obtained in the section on amino acid uptake, it, therefore, appears that this may become the only basis on which the results can be given a general consideration.

No problem is more fundamental to biology than that of the passage of materials into and out of living cells. The problem is related to the properties of the cell wall which are determined by factors such as the size and shape of the micelle of the cell wall, the electric charge of the cell, the pH of the surrounding medium and the metabolic activity or the age of the cell. The whole field is not very well known. Most of the data are obtained from artificial membranes since the nature of the cell membrane itself so far has defied direct observations in living cells.

As to differences in uptake of amino acids by lactic acid bacteria, this is now a well established phenomenon which is receiving more consideration in problems concerning amino acid nutrition. The suggested theory that the uptake is regulated by the presence of other amino acids is supported by very little direct evidence. It is felt, however, that the amino acid interrelationships discussed in this work may be taken as indirect evidence for such a statement, and if this is true, the whole picture is considerably clarified.

As Christensen et al (98) showed for the mouse carcinoma cells, the effect of the other amino acids in the medium may be either to inhibit or to stimulate the uptake of the limiting amino acid. Thus in a normal medium, the 17 amino acids present probably compensate each other's effects so that the amount of stimulation is equal or near equal to the amount of inhibition, and the limiting amino acid is allowed to enter the cell. Only in one instance is such an equilibrium not the case under the general conditions employed in all this work. That is in amino acid inhibition of glutamic acid utilization by Lactobacillus arabinosus at pH 7. Here the sum of inhibitory effects seem to be too great to allow glutamic acid to enter the cell.

Considering the four different classes of effects of lowering the amino acids in the medium in the order they are discussed previously, the following mechanisms, based on the theory of the interference with uptake, can be proposed:

1a. The inhibition of utilization of a limiting amino acid by high amount of another amino acid in the medium might be a situation in which uptake of the limiting amino acid is inhibited by the other amino acid.

When the concentration of the inhibiting amino acid is lowered, the inhibition would be reduced and the passage of the limiting amino acid into the cell would take place more readily.

1b. The inhibition of utilization of the limiting amino acid by low amount of another amino acid in the medium would similarly be the case of one amino acid stimulating the uptake of another amino acid. If this stimulation were decreased by lowering the concentration of the stimulatory amino acid, the uptake of the limiting amino acid would be correspondingly reduced. This does not necessarily mean that the stimulatory amino acid is required under all conditions in order to accomplish uptake of the other. It may only mean that in the presence of other amino acids the stimulation is necessary to compensate for a possible inhibition by these other amino acids. In other words, it is difficult to say whether or not the stimulating amino acid would be required under all conditions, as, for example, when the medium contains the limiting amino acid alone.

II a and b. The inhibition of synthesis of the limited amino acid by high and low amounts, respectively, of another amino acid must be considered as direct consequences of the two previous cases. Here, however, rather than the limiting amino acid itself being involved, the uptake of its precursor or precursors may be disturbed. The precursors, of course, would also become limiting amino acids in this case. In the very last case, the inhibition of synthesis by "low amount," the possibility should be considered that this effect may be the direct result of lowering the concentration of the precursor itself.

The interesting effect obtained with peptides would simply indicate that peptides are taken up more readily than the free amino acids. This

is not an original thought, since other workers seem to recognize it as a general fact (39). The observation that apparently any glycine containing peptide overcomes the arginine requirement in the glycine-arginine interdependence indicates that arginine is not required as part of the peptide. This would essentially be in disagreement with the "difficult peptide synthesis theory" which was proposed for the original arginine-proline interdependence. It seems that the only effect of arginine, therefore, might be to stimulate uptake of glycine. When peptide-bound glycine is used, the requirement for this stimulation would be reduced since the peptide can enter the cell more readily than the free amino acid.

The effect of pH can also be explained from the standpoint of amino acid uptake. The observations in this work that pH 6 seems to be more optimal for growth of all the lactic acid bacteria than pH 7, may only mean that the cell wall is more easily traversed at that lower pH. If this is so, and one set of data from the uptake experiment indicates that it may be, this would explain why all the effects studied in this work are much more pronounced at pH 7. One of the outstanding features of the living cell is its high electrical resistance. This indicates that ions enter and leave living cells with difficulty, and that pH would be important for the passage of the amino acids.

Inorganic ion concentration may also be involved in connection with pH. The pH of the external medium is not the same as that in close proximity to the cell surface because of the electrical charge the cell carries. The cells in question are mostly electronegative and tend to attract protons, thereby decreasing the pH on the surface. These pH differences are very sensitive to electrolytes, since the protons may be replaced by other cations in the vicinity of the cell, and since this in turn will antagonize the establishment of the pH difference.

The fact that the "all-K medium, which has been used consistantly in this work, has proven to be more optimal in most cases than the normal medium containing both Na and K is probably connected with this problem. Other work performed in this laboratory has revealed a very sensitive relationship between pH and the concentration of K and Na in the medium. In connection with the ion involvement can be mentioned the generally-accepted theory that the ratio of monovalent cations to divalent cations is essential to the sensitivity of the phase reversibility of the oil-in-water and water-in-oil emulsions in the cell wall. The phase of this emulsion would naturally determine the kind of compounds which can pass through the cell wall.

The mechanism by which the other amino acids influence the uptake of the limiting one, and by which the uptake is accomplished is difficult to visualize. The inhibition may be a case of two amino acids being taken up at the same rate, and the one, by virtue of its higher concentration, producing an unfavorable gradient before sufficient amounts of the limiting amino acid have entered the cell. This would hold in the cases of alanine and glycine, and glutamic acid and aspartic acid, both being pairs of very similar amino acids which probably would enter the cells with the same ease. The stimulatory effects are still more difficult to explain. They seem to indicate a more specific relationship between the stimulatory and the limiting amino acid.

The whole study of amino acid uptake by living cells is still only in a very early developing stage. Its connection with cellular nutrition may very well prove to be a close one, and many of the problems related to the metabolism in the cells may be solved by further studies of properties of the cell wall and mechanisms by which different materials pass

through it. Undoubtedly, such studies will involve several factors which have been underestimated or neglected in the earlier work, such as pH, ion concentration, red ox potential, etc. Many of these studies will probably shift more and more over to the biophysicist, but the biochemical methods employed in this work are far from exhausted as sources for information. Work with microbiological assay techniques will undoubtedly continue to yield significant contributions to our information about this very fascinating problem.

LITERATURE CITED

1. Baribo, L. E. and E. M. Foster, J. Dairy Sci., 34: 1136, (1951).

2. Hirsch, A. and D. M. Wheater, J. Dairy Research, 18: 193, (1951).

3. Wheater, M. D., A. Hirsch and A. T. R. Mattick, Nature, 168: 659, (1951).

4. Snell, E. E. and F. M. Strong, Ind. Eng. Chem. Anal. Ed., 11: 346, (1939).

5. Dunn, M. S., Physiol. Rev. 29: 219, (1949).

6. Schweigert, B. S. and E. E. Snell, Nutrition Abstracts and Revs., 16: 497, (1947).

7. Dunn, M. S., Food Technol., 1: 269, (1947).

8. Knight, B. C. J. G., Vitamins and Hormones, Vol. III, pp. 105. Academic Press Inc., New York, N. Y. (1945).

9. Orla-Jensen, S., Mem. acad. roy. sci. Danmark. Sect. sci. 8me, ser., 5: 81, (1919).

10. Peterson, W. H. and M. S. Peterson, Bact. Revs. 9: 49, (1945).

11. Snell, E. E., Wallerstein Labs. Communs., 11: 81, (1948).

12. Snell, E. E., Vitamin Methods, Vol. I, pp. 327, Academic Press Inc., New York, N. Y. (1950).

13. Snell, E. E., Bact. Revs., 16: 235, (1952).

14. Hendlin, D., Bact. Revs., 16: 241, (1952).

15. Gest, H. and J. O. Lampen, J. Biol. Chem., 194: 555, (1952).

16. Gunsalus, I. C. and M. Gibbs, J. Biol. Chem. 194: 871, (1952).

17. Lyman, C. M., O. Moseley, S. Wood, B. Butler and F. Hale, J. Biol. Chem. 167: 177, (1947).

18. Lardy, H. A., R. L. Potter and R. H. Burris, J. Biol. Chem., 179: 721, (1949).

19. Snell, E. E., Bacterial Physiology, pp. 214, Academic Press Inc., New York, N. Y. (1951).

20. Snell, E. E., Plant growth substances, pp. 431, University of Wisconsin Press, Madison, Wis. (1951).

21. Broquist, H. P. and E. E. Snell, J. Biol. Chem. 188: 431 (1951).

22. Snell, E. E. and H. K. Mitchell, Proc. Natl. Acad. Sci. U. S., 27: 1, (1941).

23. Hendlin, D., M. C. Caswell, V. J. Peters and T. R. Wood, J. Biol. Chem. 186: 647, (1950).

24. Snell, E. E., and B. M. Guirard, Proc. Natl. Acad. Sci. U. S., 29: 66, (1943).

25. Holden, J. T., C. Furman and E. E. Snell, J. Biol. Chem. 178: 789, (1949).

26. Holden, J. T. and E. E. Snell, J. Biol. Chem. 178: 799, (1949).

27. Holden, J. T., R. B. Wildman and E. E. Snell, J. Biol. Chem. 191: 559, (1951).

28. Skeggs, H. R., H. M. Nepple, K. A. Valentik, J. W. Huff and L. D. Wright, J. Biol. Chem. 184, 211, (1950).

29. Snell, E. E., E. Kitay and W. S. McNutt, J. Biol. Chem. 175: 473, (1948).

30. Wright, L. D., H. R. Skeggs and J. W. Huff, J. Biol. Chem. 175: 475, (1948).

31. Bardos, T. J., T. J. Bond, J. Humphreys and W. Shive, J. Am. Chem. Soc., 71: 3852, (1949).

32. Berkinshaw, J. H., H. Raistrick and G. Smith, Biochem. J. 36: 823 (1942).

33. Brickson, W. L., L. M. Henderson, I. Solhjell and C. A. Elvehjem, J. Biol. Chem. 176 : 517, (1948).

34. Meinke, W. W. and B. R. Holland, J. Biol. Chem. 173: 535, (1948).

35. Sirny, R. J., L. T. Cheng and C. A. Elvehjem, J. Biol. Chem. 190: 547, (1951).

36. Snell, E. E. and H. Kihara, J. Biol. Chem. 197: 783, (1952).

37. Miller, H. K. and H. Waelsch, Arch. Biochem. and Biophys., 35: 184, (1951).

38. Kihara, H. and E. E. Snell, J. Biol. Chem., 197: 791, (1952).

39. Kihara, H., O. Klatt and E. E. Snell, J. Biol. Chem. 197: 801, (1952).

40. Klungsöyr, M., R. J. Sirny and C. A. Elvehjem, J. Biol. Chem., 189: 557, (1951).

41. Krehl, W. E., J. S. Fruton, J. Biol. Chem., 173: 479, (1948).

42. Agren, G., Acta. Physiol. Scand., 13: 347, (1947).

43. Agren, G., Acta. Chem. Scand., 2: 611, (1948).

44. Meinke, W. W. and B. R. Holland, J. Biol. Chem., 184: 251, (1950).

45. MacLeod, R. A. and E. E. Snell, Ann. N. Y. Acad. Sci., 52: 1249, (1950).

46. Sirny, R. J., Doctor Dissertation, University of Wisconsin, (1951).

47. MacLeod, R. A. and E. E. Snell, J. Bact., 59: 783, (1950).

48. Price, S. A., and H. C. H. Graves, Nature, 153: 461, (1944).

49. Borek, E. and H. Waelsch, J. Biol. Chem., 190: 191, (1951).

50. Lowry, O. H. and O. A. Bessey, J. Biol. Chem., 155: 71, (1944).

51. Bohonos, N., B. L. Hutchings and W. H. Peterson, J. Bact., 44: 479, (1942).

52. Hendlin, D. and M. H. Soars, J. Biol. Chem., 188: 603, (1951).

53. Kocher, V., Intern. Z. Vitaminforsch., 20: 369, (1949).

54. Koditschek, L. K., D. Hendlin and H. B. Woodruff, J. Biol. Chem., 179: 1093, (1949).

55. Evans, R. J. and H. A. Butts, Science, 109: 569, (1949).

56. Rose, D. and R. Peterson, Canadian J. Research, B 27: 428, (1949).

57. Friedman, L. and O. L. Kline, J. Biol. Chem., 184: 599, (1950).

58. Friedman, L. and O. L. Kline, J. Nutr. 40: 295, (1950).

59. Haugaard, G., L. Tumerman and H. Silvestri, J. Am. Chem. Soc., 73: 5494, (1951).

60. Maillard, L. C., Compt. rend. 154: 66, (1912).

61. Orla-Jensen, A. D., J. Soc. Chem. Ind. (London), 52: 374T, (1933).

62. Rabinowitz, J. C. and E. E. Snell, J. Biol. Chem., 169: 631, (1947).

63. Smily, K. L., D. F. Niven and J. M. Sherman, J. Bact. 45: 445, (1943).

64. Henderson, L. M. and E. E. Snell, J. Biol. Chem., 172: 15, (1948).

65. Stele, B. F., H. E. Sauberlich, M. S. Reynolds and C. A. Bauman, J. Biol. Chem., 177: 533, (1949).

66. Meinke, W. W. and B. R. Holland, J. Biol. Chem., 184: 251, (1950).

67. Dunn, M. S., L. E. McClure and R. B. Merrifield, J. Biol. Chem., 179: 11, (1949).

68. General Information for the Use of the Cannon Automatic Dispensor and Titrator. International Instrument Co., Los Angeles, Calif.

69. Hac, L. R., E. E. Snell and R. J. Williams, J. Biol. Chem., 159: 273, (1945).

70. Lyman, C. M., K. A. Kuiken, L. Blotter and F. Hale, J. Biol. Chem., 157: 395, (1945).

71. Baumgarten, W., A. N. Mather and L. Stone, Cereal Chem. 22: 514, (1945).

72. Winnick, T., I. Moring-Claesson and D. M. Greenberg, J. Biol. Chem., 175: 121, (1948).

73. Siekevitz, P., T. Winnic and D. M. Greenberg, Federations Proc. 8: 250, (1949).

74. Sakami, W., J. Biol. Chem., 176: 995, (1948).

75. Sakami, W., Federation Proc. 8: 246, (1949).

76. Shemin, D., J. Biol. Chem., 162: 297, (1946).

77. Lascelles, J. and D. D. Woods, Nature, 166: 649, (1950).

78. Gale, E. F., J. Gen. Microbiol., 1: 53, (1947).

79. Gale, E. F. and E. S. Taylor, J. Gen. Microbiol., 1: 77, (1947).

80. Taylor, E. S., J. Gen. Microbiol., 1: 86, (1947).

81. Gale, E. F. and P. D. Mitchell, J. Gen. Microbiol., 1: 299, (1947).

82. Gale, E. F., and E. S. Taylor, J. Gen. Microbiol., 1: 314, (1947).

83. Gale, E. F., J. Gen. Microbiol., 1: 327, (1947).

84. Gale, E. F., J. Gen. Microbiol. 2: iv, (1948).

85. Gale, E. F. and A. W. Rodwell, J. Gen. Microbiol., 3: 127, (1949).

86. Gale, E. F., J. Gen. Microbiol., 3: 369, (1949).

87. Najjar, V. A. and E. F. Gale, Biochem. J., 46: 91, (1950).

88. Gale, E. F., Biochem. J., 48: 286, (1951).

89. Gale, E. F., Biochem. J., 48: 290, (1951).

90. Gale, E. F. and T. F. Paine, Biochem. J. 48: 298, (1951).

91. Gale, E. F. and M. B. van Halteren, Biochem. J. 50: 34, (1951).

92. Gale, E. F., Biochem. J. 47: xxvi, (1950).

93. Gale, E. F., J. Gen. Microbiol., 4: v, (1950).

94. Gale, E. F., Bull. Johns Hopk. Hosp. 83: 119 (1948).

95. Christensen, H. N., T. R. Riggs and N. E. Ray, J. Biol. Chem., 194: 41, (1952).

96. Riggs, T. R., H. N. Christensen and I. M. Palatine, J. Biol. Chem., 194: 53, (1952).

97. Christensen, H. N. and T. R. Riggs, J. Biol. Chem., 194: 57, (1952).

98. Christensen, H. N., T. R. Riggs, H. Fischer and I. M. Palatine, J. Biol. Chem., 198: 1, (1952).

99. Christensen, H. N. T. R. Riggs, H. Fischer and I. M. Palatine, J. Biol. Chem., 198: 17, (1952).

APPENDIX

A. Media for Storage and Transfer of Organisms.

Agar medium:

Yeast extract	10.0 gm.
Glucose	2.5 gm.
Agar	15.0 gm.
K-acetate	5.0 gm.
Water to 1000 ml.	

Liquid transfer medium:

Glucose	1.0%
K-citrate	1.0%
K-acetate	0.1%
K_2HPO_4	0.5%
NH_4Cl	0.3%
Tryptone	0.5%
Yeast extract	0.5%
Salts C soln.*	1.0%
Vitamin soln.*	0.5%

Dissolved in water, and pH adjusted at 6.0

The media were sterilized and stored in the refrigerator.

B. Basal Media for Microbiological Assays.

Amino acid mix**(for 100 tubes at 2 ml. final assay volume):

DL-Alanine	200 mg.	DL-Threonine	40 mg.
DL-Aspartic acid	200 mg.	DL-Tryptophan	40 mg.
L-Glutamic acid	200 mg.	DL-Valine	40 mg.
L-Arginine.HCl	40 mg.	Glycine	20 mg.
DL-Isoleucine	40 mg.	L-Cystine	20 mg.
L-Lysine.HCl	40 mg.	L-Histidine.HCl	40 mg.
DL-Methionine	40 mg.	L-Leucine	20 mg.
DL-Phenylalanine	40 mg.	L-Proline	20 mg.
DL-Serine	40 mg.	L-Tyrosine	20 mg.
		Made up to 50 ml. with acid and heat.	

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* Composition given in Appendix B.
**The amino acid assayed for, to be omitted.

B. (Continued)

Sugar mix for L. brevis (for 100 tubes, at 2 ml. final assay volume):

L-Arabinose	4.0 gm.
Glucose	0.6 gm.
K-Acetate	4.0 gm.
K_2HPO_4	1.0 gm.
NH_4Cl	0.6 gm.
Salts C soln.	4.0 ml.
Vitamin soln.	2.0 ml.
AGU-soln.	2.0 ml.
X-soln.	2.0 ml.

50 ml. of the amino acid mix added as described below.

Sugar mix (for 100 tubes at 2 ml. final assay volume):

Glucose	4.0 gm.
K-citrate.H_2O	4.4 gm.
K-acetate (anhydr.)	0.2 gm.
NH_4Cl	0.6 gm.
K_2HPO_4	1.0 gm.
Salts C soln.	4.0 ml.
AGU-soln.	2.0 ml.
X-soln.	2.0 ml.
Vitamin soln.	2.0 ml.

50 ml. of amino acid mix is added, and the total made up to 100 ml. pH adjusted to the desired value.

Solutions for the above sugar mix:

Salts C

$FeSO_4.7H_2O$	0.5 gm.
$MnSO_4.7H_2O$	2.0 gm.
$MgSO_4.7H_2O$	10.0 gm.

Dissolved with the aid of HCl, and made up to 250 ml.

AGU-soln.

Adenine-sulphate	250 mg.
Guanine, HCl	250 mg.
Uracil	250 mg.

Dissolved with the aid of HCl and made up to 250 ml.

Vitamin soln.

Thiamin	25.0 mg.
Niacin	25.0 mg.
Ca-panto-thenate	25.0 mg.
Pyridoxal	5.0 mg.
Riboflavin	25.0 mg.

(continued)

X-soln.

Xanthine	250 mg.

Dissolved in dilute KOH and made up to 250 ml.

B. (Continued)

Vitamin soln.

PABA	5.0 mg.
Biotin*	0.25 mg.
Folic acid**	0.25 mg.

Riboflavin dissolved first with hot water and acid, then the rest of the vitamins added and volume made up to 250 ml.

C. Buffer-Salt Solution for the Amino Acid Uptake Experiments.

"Gale's solution":

0.1%	KH_2PO_4
0.33%	Na_2HPO_4
0.1%	NaCl
0.07%	$MgSO_4.7H_2O$

pH adjusted to the desired value.

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* Biotin stored in soln. in 50% EtOH.
**Folic acid stored in soln. in dil. KOH in 50% EtOH.

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